Proceedings
of the
Society
for
Experimental Biology and Medicine

VOL. 83 JUNE, 1953 No. 2

SECTION MEETINGS

CLEVELAND, OHIO	
University Hospitals	May 14, 1953
IOWA	
State University of Iowa	May 6, 1953
State University of Iowa	May 26, 1953
MARYLAND	
Johns Hopkins University	May 20, 1953
MINNESOTA	
University of Minnesota	May 20, 1953
OHIO VALLEY	
University of Cincinnati	May 23, 1953
WESTERN NEW YORK	
Syracuse University	May 9, 1953

Relation Between Pressure and Concentration Difference Across Membranes Permeable to Solute and Solvent.* (20306)

EUGENE GRIM. (Introduced by M. B. Visscher.)
From the Department of Physiology, University of Minnesota, Minneapolis.

Most of the osmotic systems encountered in biology involve membranes that are permeable to solute as well as to solvent molecules, hereinafter called leaky membranes. In the study of material transport across such membranes, the relation between the solute concentration difference and the hydrostatic pressure difference across the membrane at any time is of fundamental importance. The purpose of this note is to demonstrate that this relation is not given by the classical van't Hoff osmotic pressure equation. In 1887, van't Hoff(1) pointed out that the perfect gas law could be applied directly to the observations of Pfeffer on the equilibrium hydrostatic pressure difference developed across the nearly semipermeable copper-ferrocyanide membrane when that membrane separated a solution of a non-electrolyte from water. In any system with a strictly semipermeable membrane (a membrane permeable to solvent only) separating solutions of non-electrolytes, the hydrostatic pressure difference, Δp, existing across the membrane *at equilibrium* (by definition, the osmotic pressure difference, $\Delta\pi$) is directly proportional to the difference in activity, or neglecting activity coefficients, the difference in concentration, Δc, of the solute in the two solutions separated by the membrane. The proportionality factor in this relation is RT, where R is the gas constant and T is the absolute temperature. The "van't Hoff relation" describing the equilibrium state is thus given by the equation:

$$\Delta p_{eq} = \Delta\pi = \Delta c_{eq}RT \qquad (1)$$

In systems in which the membrane is permeable, with various degrees of restriction, to molecules of the solute as well as to solvent molecules, it is the changes in Δp and Δc occurring during the approach to equilibrium rather than the equilibrium state itself which is of major interest; for, at equilibrium the composition of the two solutions separated by the membrane have become identical through interdiffusion; *viz.*, Δp and Δc equal zero. Even in the simplest case involving aqueous solutions of non-electrolytes (the one to be considered here) the relation between Δp and Δc at any time preceding equilibrium is more complex than that expressed by equation(1).

---

* This work was carried out with the aid of a grant from the Life Insurance Medical Research Fund.

Two cases will be considered here. The first applies to a system in which the volumes of the two solutions separated by a leaky membrane are maintained constant by changes in the pressure difference across the membrane to compensate for the changing solute concentration difference. The second case is concerned with systems in which the pressure difference is maintained constant and equal to zero and the volumes of the solutions change with time. Recently, the mathematical relation between concentration and pressure difference across a leaky membrane separating a non-electrolyte solution from water has been developed by Laidler and Schuler (2). Their general equation (II-5), with appropriate changes in symbols to agree with those used here, is:

$$\frac{dV_i}{dt} = K\left[\frac{Q_1 V_1^2}{RT}(\Delta c_2 RT - \Delta p) - \frac{Q_2 V_1 V_2}{RT}(\Delta c_2 RT)\right] \quad (2)$$

where the subscript i refers to the solution inside a test-tube shaped membrane and the subscripts 1 and 2 refer to water and non-electrolyte, respectively. The new symbols have the following meaning: K is essentially a constant for any given membrane, being dependent upon the total area, A^m, and the thickness, Δx, of the membrane. Q is the permeation coefficient, has the same units as the diffusion coefficient, D, and may be considered equal to the product of D and the distribution ratio for the particular molecular species in question at the interface between membrane and solution. As pointed out by Laidler and Schuler, this ratio may be considered to be qualitatively equal to the ratio of pore area available for diffusion of the particular molecular species to the total membrane area; *i.e.*, A^p/A^m. V is the partial molar volume.

For case I (*i.e.*, $dV_i/dt = 0$), this equation reduces to:

$$Q_1 V_1 (\Delta c_2 RT - \Delta p) - Q_2 V_2 (\Delta c_2 RT) = 0 \quad (3)$$

Rearranging and collecting terms:

$$\Delta p = \left(1 - \frac{Q_2 V_2}{Q_1 V_1}\right)\Delta c_2 RT = k\Delta c_2 RT = k\Delta\pi \quad (4)$$

Thus a new proportionality factor which will be called k is added to van't Hoff's relation when applied to leaky membranes, the factor being dependent on the diffusion coefficients, partial molar volumes, and the pore areas available for diffusion of the solute and solvent molecules. Qualitatively, equations (2), (3), and (4) are merely statements of the fact that the volume and/or pressure changes on both sides of the membrane are dependent upon solute as well as solvent transport across the membrane. In the special case in which the membrane is semipermeable (Q_2 equals zero), equation (4) reduces to the classical van't Hoff equation; *e.g.*, in the case of plasma proteins and the capillary membrane. For the usual case with non-electrolytes and biological membranes, Q_2V_2 is smaller than Q_1V_1; hence $k < 1$ and $\Delta p < \Delta\pi$. Theoretically, of course, k may equal zero or be greater than one. The error introduced in calculating Δp from a measured Δc or vice versa by means of the van't Hoff equation will depend on the value of k and as will be shown later can easily be as great as 2 or 3 orders of magnitude.

For case II (*i.e.*, $\Delta p = 0$), equation (2) reduces to:

$$\frac{dV_i}{dt} = K\Delta c_2 (Q_1 V_1^2 - Q_2 V_1 V_2) \quad (5)$$

The volume change rate or "osmotic transport rate" is a function of the nature of the non-electrolyte as well as the solute concentration difference; and depending on the non-electrolyte, it may be positive, negative, or zero. With a semipermeable membrane in such a system ($Q_2 = 0$), the magnitude of the osmotic transport rate becomes for a given membrane a function of the concentration difference only and is independent of the nature of the non-electrolyte (as might be predicted from an extrapolation of the van't Hoff relation to such systems). This has been shown experimentally by Berkeley and Hartley(3). If, in case II, the outside compartment is filled with solution of a non-electrolyte different from but at the same concentration as the inside non-electrolyte, then, with a semipermeable membrane, it would be found that the net volume change of the inside solution would be zero, since the osmotic transport rate is a function of the concentration difference only. With a leaky membrane, however, it would be expected that a net volume change would occur, since the *QV* values of the two different non-electrolytes would most likely be unequal. Any net osmotic transport at zero time in such a system with two iso-osmotic solutions obviously could not be explained by an extrapolation of the van't Hoff law.

Experimental. The first experiments are concerned with a system treated as case I in the preceding theoretical discussion. It provides a direct comparison between the observed hydrostatic pressure difference across the membrane required to maintain the isovolumetric state and the osmotic pressure calculated by the van't Hoff equation from the measured concentration difference existing across the membrane at the same time. For such an experiment, the procedure was as follows: Test-tube shaped unoxidized collodion membranes of 35 mm diameter and 95 mm length were prepared by the method of Sollner and collaborators(4). A typical specimen was tied to a Lucite stopper which was attached to a capillary manometer of approximately 0.5 mm diameter. The membrane was filled with a measured volume of glucose solution of known concentration through a small hole in the Lucite stopper which was subsequently sealed. The membrane was then immersed in a measured volume of water. Stirring of the solutions on either side of the membrane was initiated immediately, the outside solution by two streams of water-saturated oxygen and inside by means of two small Alnico magnets, magnetically rotated on axes attached to a rod suspended from the Lucite stopper into the center of the inside solution. By means of a syringe in communication with the manometer system through a side arm just above the junction of the manometer and Lucite stopper, the pressure inside the membrane was increased as rapidly as possible until the liquid level in the manometer remained at a constant height, showing that the hydrostatic pressure difference across the membrane was just sufficient to prevent any net movement of liquid across the membrane. The height and time (usually one to two minutes after the initial immersion) was noted. At frequent intervals during a subsequent period of 2 to 3 hours the height and time were again noted. Simultaneous with each of several of these readings, exactly one milliliter was pipetted from the outside solution and subjected to analysis for its glucose content. From the concentration of glucose in the outside solution, it is possible to obtain the concentration inside by difference; hence the concentration difference, $\triangle c$, can be determined. From the values of $\triangle c$, $\triangle \pi$ was calculated by means of the van't Hoff equation.

In Fig. 1, the results of such an experiment

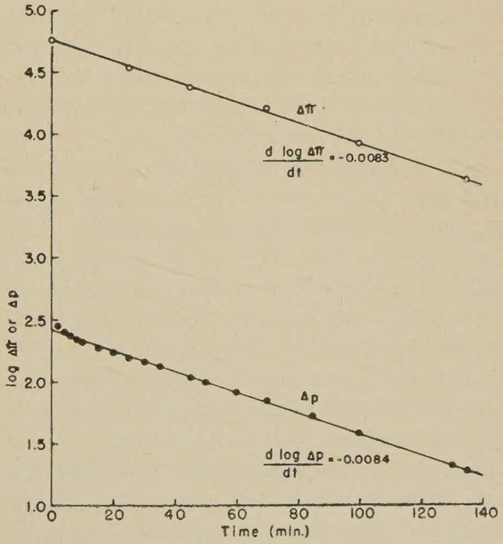


FIG. 1. Relation between observed and "van't Hoff" hydrostatic pressure differences across a leaky membrane.

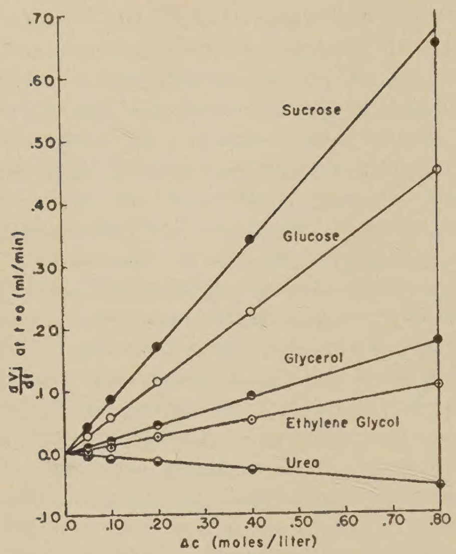


FIG. 2. Influence of concentration difference and nature of the solute on osmotic transport rates across leaky membranes.

are shown. The upper curve is a plot of log ($\Delta\pi$), the pressure difference calculated from the van't Hoff equation, versus time; and the lower curve is a similar plot of log (Δp), the observed pressure difference. Each set of values falls on a straight line as is to be expected for a diffusion process. Of primary importance is that at all times, Δp is much smaller than $\Delta\pi$. Furthermore, as expected from equation (4), the Δp values are directly proportional to the $\Delta\pi$ values as indicated by the nearly equal slopes of the 2 curves; *i.e.*, $\Delta p = k\Delta\pi$ with k in this particular case approximately equal to 0.0046.

The following experiments are concerned with case II and were made in some earlier work of the author(5) performed for a different purpose. In the first group of experiments, the hydrostatic pressure difference was maintained essentially constant and approximately equal to zero, the volume of the solution inside the membrane varying with time. By observing the initial rate of volume change of the inside solution associated with the initial known concentration difference, the relation between the magnitude of the osmotic transport rate across the membrane and the concentration difference, as well as the nature of the non-electrolyte is obtained. In Fig. 2, the results of such experiments are shown, the initial rate of volume change of the inside solution being plotted against the initial concentration difference. For a given non-electrolyte the rate of volume change is directly proportional to the concentration difference as expected from equation (5). Further, the volume change rates are different for the various non-electrolytes at the same concentration difference, an occurrence expected from the inequality of the values for Q_2V_2. Of particular interest is the case of urea in which the sign of the volume change rate is opposite to that of the concentration difference; *i.e.*, negative osmosis occurs, the net movement of liquid being from the solution to the solvent side of the membrane. The explanation of the latter case, on the basis of the theoretical discussion, is that Q_2V_2 is greater than Q_1V_1; *i.e.*, a greater volume of urea than water is transported per unit time across the membrane.

In the second group of experiments associated with case II, the outside compartment was filled with an iso-osmotic solution of a different non-electrolyte instead of distilled water. The results are shown in Table I and demonstrate that, contrary to what would be expected on the basis of an extrapolation of the van't Hoff relation; there is a net movement of solution across the membrane despite the zero concentration difference. Of especial interest again is the observation that the osmotic transport rate is greater in the system in which the outside compartment contains urea solution instead of distilled water.

TABLE I. Osmosis in Iso-Osmotic Systems.

Solute in the inside solution	Solute in the outside solution	$\frac{dV_1}{dt}$ (ml/min.)
Sucrose	Glucose	.11
"	Glycerol	.23
"	Ethylene glycol	.26
"	Distilled water	.31
"	Urea	.35

Discussion. The preceding observations have important implications in relation to the conclusions of a recent paper by Pappenheimer, Renkin, and Borrero(6) on material transport across the leaky capillary membrane. These workers have introduced a method for determining the area available for diffusion for various molecules per unit path length through the capillary wall. The method involves the measurement, for a test molecule, of the variables in the Fick equation:

$$\frac{dm}{dt} = -DA^p \cdot \frac{\Delta c}{\Delta x} \qquad (6)$$

where m is the net amount of test molecule crossing the membrane and the other symbols denote the same quantities as earlier in this paper. Substituting these values into the equation, a value for $A^p/\Delta x$, the diffusion area per unit path length is obtained.

In their experiments, the test molecule is introduced into the perfusing fluid of an isolated cat limb, and after a short time interval, the perfusing fluid flow rate and the arteriovenous difference of concentration are measured. From these values the quantity, dm/dt is readily obtained. Unfortunately, the quantity, Δc, is not obtained by direct measurement. Their procedure is to measure the increase in the mean capillary hydrostatic pressure required to prevent movement of fluid from the interstitial spaces into the capillaries as a result of the "partial osmotic pressure" exerted by the added test molecules; *i.e.*, the pressure increase necessary to maintain the isovolumetric state (see case I above).[†] The value for this pressure increment is then erroneously substituted into the van't Hoff equation as being equal to the "increase in osmotic pressure due to the added test molecules", and the equation is solved for Δc. By this means, they arrive at the following equation for the diffusion area per unit path length:

$$\frac{A^p}{\Delta x} = \frac{Q_b (c_a - c_v) RT}{D \cdot \Delta p} \qquad (7)$$

The correct equation, taking cognizance of the permeability of the capillary membrane to the test molecules involved should include the factor, k, in the numerator of the right-hand side of equation (7). All of the values given in the Pappenheimer paper for $A^p/\Delta x$ are, thus, too large by a factor which must be determined experimentally for each test molecule with the capillary membrane. The magnitude of the error might be estimated from the data obtained with the collodion membranes of this study. These artificial membranes are similar in many properties to the capillary wall. They are impermeable to serum albumin and slowly permeable to oxyhemoglobin. They have a filtration coefficient of approximately 2 x 10^{-5} ml/min/mm Hg/cm^2 as compared to filtration coefficients calculated from Pappenheimer's data for the capillary wall in the range of 2.5 x 10^{-4} to 2.3 x 10^{-6}. An important difference, of course, is that the collodion membranes used here are approximately 100 times as thick as the capillary wall (100 μ as compared to 1 μ); however, this difference is partially counterbalanced by the fact that A^p/A^m for the collodion membranes used here is of the order of 0.2 which is much larger than similar values for the capillary wall. On the basis of approximate comparability of the two kinds of membranes, Pappenheimer's values are too large by approximately two orders of magnitude. Such errors could conceivably be relevant to Pappenheimer's criticism of the work of Flexner, Cowie, and Vosburgh(7) made on the basis that his (Pappenheimer's) $A^p/\Delta x$ values were appoximately 200 times larger than similar values calculated from the data of Flexner *et al.*

A note of caution in the study of transcapillary diffusion of electrolytes may be appropriate here. In the case of electrolyte solutions and membranes bearing fixed dissociable groups, the factor, k, becomes an unknown function of the epsilon-potential across the membrane and the zeta-potential on the membrane, as well as a function of Q and *V*. It has been shown experimentally by the author that with certain electrolyte solutions k may even exceed one; *i.e.*, $\Delta p > \Delta \pi$.

Summary. 1. Experiments are presented which demonstrate, as expected from theoretical considerations, that the classical van't Hoff equation cannot be employed to ascertain the relation between pressure difference and concentration difference across a membrane in the dynamic situation existing in osmotic systems involving membranes permeable to solute as well as to solvent. It is shown that the pressure difference necessary to prevent a net osmotic movement of fluid across a leaky membrane may be smaller by several orders of magnitude than the pressure difference necessary to accomplish the same effect with a semipermeable membrane; *i.e.*, a membrane to which the van't Hoff equation applies. It is also shown that, contrary to what would be expected on the basis of reasoning from the van't Hoff equation, the osmotic transport rate across a leaky membrane is a function not only of the solute concentration difference but also of the nature of the solute molecules. Further, it is demonstrated that net transport of liquid may occur across leaky membranes separating iso-osmotic solutions of different non-electrolytes. 2. In all these cases, the use of equations derived for the equilibrium state across a semipermeable membrane (*e.g.* the van't Hoff equation) would lead to decidedly erroneous results. 3. These observations demonstrate the use of van't Hoff's equation in a recent analysis of capillary permeability to be incorrect.

[†] In Pappenheimer's work it was actually the nearly equal isogravimetric state.

---

1. Van't Hoff, J. H., *Z. f. physikal. Chem.*, 1887, v1, 481.

2. Laidler, K. J. and Schuler, K. E., *J. Chem. Physics*, 1949, v17, 851.

3. Berkeley, Earl of, and Hartley, E., *Proc Roy. Soc. A*, 1909, v82, 271.

4. Carr, C. W., and Sollner, K., *J. Gen. Physiol.*, 1944, v28, 119; Gregor, H. P., and Sollner, K., *J. Phys. Chem.*, 1946, v50, 53.

5. Grim, Eugene, Ph.D. Thesis, Univ. of Minn., 1950, p247.

6. Pappenheimer, J. R., Renkin, E. M., and Borrero, L. M., *Am. J. Physiol.*, 1951, v167, 13.

7. Flexner, L. B., Cowie, D. B., and Vosburgh, G. J., *Cold Spring Harbor Symp. Quant. Biol.*, 1948, v13, 88.

Received March 20, 1953. P.S.E.B.M., 1953, v83.

Mack Virus—Serum and Gamma Globulin Neutralization of Unidentified Agent Isolated From Suspected Nonparalytic Poliomyelitis.* (20307)

Alex J. Steigman, U. Pentti Kokko, and Rosalie J. Silverberg.

From the Kentucky Child Health Foundation Research Laboratory, Department of Pediatrics, University of Louisville School of Medicine.

We wish to describe certain observations on an unclassified virus isolated from tissue-cultures of the feces of a child (Mack) who developed thermostable specific antibodies in convalescence. We believe that the child's illness was caused by the agent which is tentatively referred to as *Mack* virus. The 5-year-old girl's illness occurred during a poliomyelitis outbreak in Cincinnati during August, 1947, with symptoms of headache, pain and "stiffness" of the neck, temperature of 103°F and pleocytosis (40 leucocytes per cu mm, 65% lymphocytes.) Recovery was complete in a few days. Although objective nuchal-spinal rigidity was not detected the clinical findings were compatible with a short-lived aseptic meningitis syndrome. For additional clinical and epidemiological details the reader is referred to patient No. 7 (K.M.) included in a study reported by Sabin and Steigman(1).

Characteristics of Mack virus in tissue-culture—isolation and titration. The roller tube tissue-culture system described by Robbins, Weller, and Enders(2) for poliomyelitis virus was used with some modification. Normal rhesus and cynomolgus monkey testicles were used as the source of fibroblastic proliferation. Natural and synthetic(3) nutrient media which are used in cultivating poliomyelitis virus were used equally well in these studies. The presence of the *Mack* virus in the culture system is indicated by cytopathogenic effects upon the fibroblastic outgrowths which are specifically inhibited by appropriate

---

* Aided by a grant from the National Foundation for Infantile Paralysis.

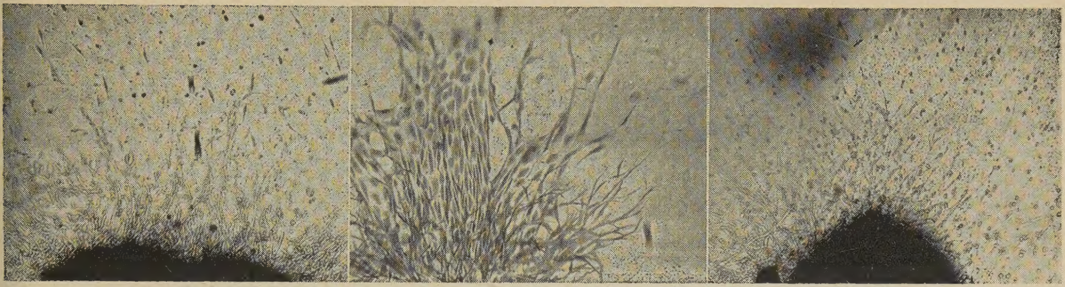


FIG. 1. Cytopathogenic effect of *Mack* virus indistinguishable from that of poliomyelitis on the fibroblastic outgrowth. To left is Type 1 poliomyelitis (Brunhilde); to right is *Mack* virus; central panel is control. Cultures were prepared simultaneously. ×70.

antisera. The character of the fibroblastic degeneration induced by the *Mack* virus cannot be distinguished morphologically from that induced by poliomyelitis. That the virus was derived from the patient and not from the tissues or natural nutrient media used was assured not only by the patient's specific serological response but also because of the ease of repeated isolation in different experiments. The virus was demonstrated readily in the fibroblastic outgrowth of the only human tissue studied—a highly anaplastic neuroblastoma removed from an infant; it failed to induce cytopathogenic effects upon cultures of adult mouse testicles. In the presence of *Mack* virus, fibroblastic degeneration appeared on the third day and was generally complete by the seventh day. Seven serial passages *in vitro* representing 17 changes of nutrient media at 4-day intervals have been made to date. Culture fluids titrated *in vitro* have an infectivity endpoint averaging $10^{-3.5}$. The endpoint is expressed as the $TCID_{50}$ (50% tissue-culture infective dose). Fig. 1 depicts the fibroblastic degeneration induced by *Mack* virus *in vitro* and the morphologically indistinguishable degeneration induced by poliomyelitis.

Neutralization of Mack virus by human sera, gamma globulin, and monkey immune serum. All sera were kept frozen except when used in tests, at which time they were heated at 56°C for 30 minutes. Acute and convalescent sera of the patient from whom the virus was recovered were diluted 5-fold in balanced salt solution and added to ±100 $TCID_{50}$ of *Mack* virus and to a member of each of the 3 known antigenic types of poliomyelitis virus. The mixtures, kept either at room temperature or at 37°C for one hour and then overnight at 4°C were inoculated into pairs of actively growing culture tubes. Controls for the sera, viruses and tissue utilized were included in the test. It will be noted that while no alteration in poliomyelitis antibody appeared, the patient's convalescent serum in a dilution of 1:1250 inhibited the fibroblastic degeneration due to *Mack* virus, whilst the 1:2 dilution of acute serum failed to do so (Table I). Specific immune serum was prepared by the intramuscular injection of a rhesus monkey with infected tissue culture fluids suspended in mineral oil adjuvants without mycobacteria(4). This serum tested *in vitro* with *Mack* virus inhibited fibroblastic degeneration when diluted 1:1250 while undiluted normal rhesus serum failed to do so. The monkey immune serum failed to influence the fibroblastic degeneration induced by members of the 3 antigenic types of poliomyelitis virus which were specifically inhibited by their own respective antisera (Table II). The sera of 6 normal adults with no known contact with *Mack* virus were tested qualitatively for neutralizing antibody; 3 of the 6 exhibited ability to neutralize ±1000 $TCID_{50}$ of *Mack* virus in tests in which 1:2 dilutions of serum were utilized.

Lyophilized pooled human gamma globulin[†] (without preservative) was redissolved and tested qualitatively for its capacity to inhibit

---

[†] Generously made available from Sharp and Dohme's Research Division by Dr. R. B. Pennell who reported that the material was processed from 9014 donors bled between May 1 and 21, 1952, in Baltimore, Boston, New York and Philadelphia.

TABLE I. Specificity of Immune Response of Patient to *Mack* Virus *In Vitro.*

Patient serum, days after onset	Serum neutralization titers in tissue-cultures of indicated viruses*†			
		Poliomyelitis type:		
	Mack	1 (Mahoney)	2 (MEF-1)	3 (Saukett)
1	1:2	<1:50	<1:50	<1:50
28	1:1250	"	"	"

* Each bearing $\pm$ 100 TCID$_{50}$ of virus.

† Final readings on 6th day when all virus control tubes showed +++ fibroblastic degeneration.

TABLE II. Failure of *Mack* Virus to be Neutralized *In Vitro* by 3 Antigenically Distinct Poliomyelitis Antisera.

Monkey antisera‡	Cytopathogenic effect of indicated viruses in tissue-cultures*†				
	Poliomyelitis type:				
	1	2	3	Mack	None
Poliomyelitis 1	0	+++	+++	+++	0
(serologic types) 2	+++	0	+++	+++	0
3	+++	+++	0	+++	0
Mack	+++	+++	+++	0	0
None	+++	+++	+++	+++	0

* Each bearing $\pm$ 1000 TCID$_{50}$ of virus.

† Final readings on 6th day when all virus control tubes showed +++ fibroblastic degeneration.

‡ Final dilution 1:4.

the fibroblastic degeneration induced by *Mack* virus *in vitro*. A 1:64 dilution of the gamma-globulin (which corresponds to a 1:3 dilution of an unconcentrated serum) when tested against decimal dilutions of *Mack* virus revealed a neutralization index of 320. In other tests using $\pm$1000 TCID$_{50}$ of virus and 1:64 dilution of gamma-globulin fibroblastic degeneration had not appeared on the 5th day by which time severe degeneration had occurred in the virus control cultures. By the 7th day degeneration became apparent in the cultures containing gamma-globulin and the $\pm$1000 TCID$_{50}$ of virus.

Animal infectivity tests. Undiluted first and third passage culture fluids of *Mack* virus inoculated cerebrally and/or subcutaneously into rabbits, guinea pigs, adult and suckling mice, and hamsters have produced no observable illness. Cortisone(5) was injected daily in the last species without evident effect. *Further tests in suckling mice.* We observed that the *stools* of the index patient failed to infect 36 suckling mice. It is also known(6) that this patient developed no neutralizing antibodies to a strain of Coxsackie virus isolated at that time and in the same locale of another patient. In view of the fecal isolation *in vitro* of an unclassified cytopathogenic virus (Weider) by Robbins *et al.*(7) and subsequently identified as Boston type Coxsackie (8), additional tests were undertaken in suckling mice; 40 others were inoculated with first and third passage tissue culture fluids of *Mack* virus, and none exhibited any illness. *Further tests in monkeys.* The patient's feces were originally described as harboring poliomyelitis virus of low pathogenicity for monkeys. This was based upon the fact that of 3 monkeys inoculated intranasally, one exhibited lesions considered to be poliomyelitis and confined to the olfactory portal(1). Tissue culture fluids were, therefore, tested by a variety of inoculation routes in monkeys, some of whom received daily intramuscular injections of cortisone. Five monkeys received repeated nasal instillations, 4 bilateral thalamic inoculations, 3 sciatic injections, 2 intraspinal injections, and 2 pharyngeal injections. All remained well for the observation period of 30 days. In further tests the patient's frozen original feces (from which the

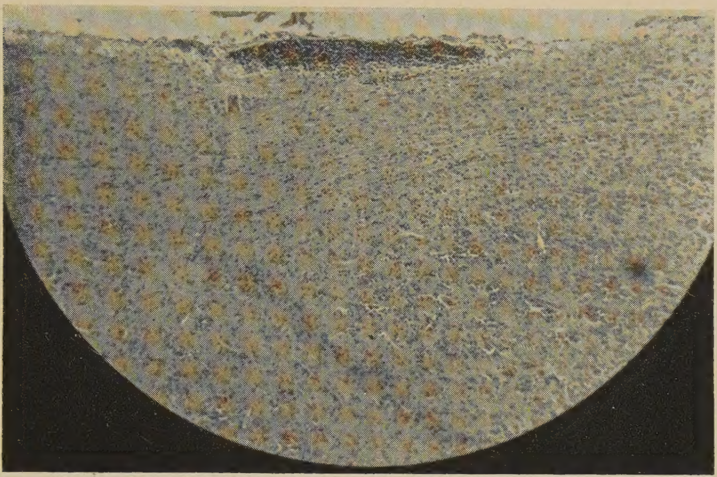


FIG. 2. Olfactory bulb of monkey inoculated nasally with *in vitro* infective *Mack* culture fluid. The round cell infiltrate shown was found on serial sections to be confined to the lamina fibrorum nervi. The mitral cell layer was normal hence not diagnostic of poliomyelitis virus. ×260.

Mack virus could be repeatedly isolated *in vitro*) were inoculated nasally and thalamically into 4 monkeys and pharyngeally into 2 others. Daily intramuscular injections of 25 mg of cortisone 2 days before and for 7-12 days after inoculation were given. These animals remained well for the observation period of 30 days.

Neurohistological studies in monkeys. The same feces which yielded *Mack* virus were earlier considered by Sabin and Steigman as containing "poliomyelitis virus of very low virulence." Their evidence was derived from serial sections of the olfactory bulbs of a monkey q.v.(1). Detailed study of CNS sections of the 22 monkeys inoculated with *Mack* virus (v. supra) revealed no lesions pathognomonic of poliomyelitis(9). Similar study of the olfactory bulbs of the 5 monkeys inoculated intranasally with tissue-culture infective fluids and the 4 additional monkeys inoculated nasally with the feces did not reveal lesions pathognomonic of poliomyelitis. The interesting lesion depicted in Fig. 2 does not meet the criteria for poliomyelitic infection of the olfactory bulbs. There is a dense ovoid infiltrate of round cells in the layer of nerve fibers but the mitral cell layer does not appear affected.

Other properties of Mack virus. The agent described passed readily through sintered glass (VF) and Seitz EK filters; was not destroyed by contact with ether and when treated with protamine was recovered in the supernatant phase. Exposure to a temperature of 56°C for 30 minutes destroyed infectivity *in vitro*. It was resistant to freezing and thawing, penicillin, and streptomycin. These properties are generally shared by the poliomyelitis and the Coxsackie viruses. Fecal specimens treated with ether and/or the antibiotics in poliomyelitis studies may be useful in revealing similar agents.

Discussion. That the patient who yielded *Mack* virus was not simultaneously infected with poliomyelitis virus is suggested by the histological study of monkeys reported herein together with failure of the patient to reveal alteration of neutralizing antibody levels to the 3 known immunological types of poliomyelitis virus in convalescence. The recovery of this unclassified virus and the appearance of specific neutralizing antibodies in convalescence should constitute noteworthy though not compelling evidence of a causal relationship. The findings herein described together with evidence of antibodies to *Mack* virus in adults and in pooled human gamma-globulin lends credence to the belief that the agent described might be parasitic in human beings. Its recovery from a febrile child with pleocytosis during the course of a poliomyelitis outbreak is not without interest. To determine the importance of this agent in disease and the types of clinical illness it might produce would require a combined clinico-epidemiologic and laboratory approach patterned after the studies of Huebner *et al.* for the Coxsackie viruses(10).

Failure to infect laboratory animals, while useful in eliminating a consideration of certain known viruses, curtails the opportunity for histopathological studies. Although no antibody studies of patients, normal individuals or gamma-globulin have been reported, it would appear that other unidentified agents cytopathogenic for human(8) and monkey (11) fibroblasts but lacking animal infectivity may be recovered from human feces. Their description suggests that the morphology of the cytopathogenicity is generally distinguishable from that of poliomyelitis virus. Another unidentified agent isolated in our laboratory produces *in vitro* morphological effects readily distinguished from those of *Mack* virus and to which it is not related immunologically(12).

The recently introduced method for the cultivation of poliomyelitis virus, by which means only *Mack* virus was discovered, may lead to the isolation of similar agents whose relationship to each other and to human disease will require elucidation. It is likely that fecal isolation attempts at virus recovery in tissue cultures will be done most commonly in association with poliomyelitis-like illnesses such as the "aseptic meningitis syndrome." The evidence reported herein would appear to exclude a concurrent infection with either poliomyelitis or Coxsackie virus in the patient. In the presence of multiple viruses in a given fecal specimen each capable of producing cytopathogenic effects the situation becomes more perplexing. Although only a few Coxsackie viruses cause fibroblastic degeneration (11), many strains of poliomyelitis virus have this property, as does the *Mack* virus. The individual identification of multiple cytopathogenic viruses simultaneously present in a given fecal specimen might be influenced by their relative ascendancy of growth curves or by the interference phenomenon and deserves study.

Summary. 1. A virus (*Mack*) has been isolated *in vitro* from a patient's feces previously reported as containing poliomyelitis virus of very limited pathogenicity for monkeys. The virus is immunologically distinct from poliomyelitis, and does not produce pathognomonic lesions of poliomyelitis in monkeys. The patient failed to develop a rise of poliomyelitis neutralizing antibodies in convalescence. 2. The virus has been cultivated in monkey and human tissue cultures in which is produced fibroblastic degeneration morphologically indistinguishable from that caused by prototype poliomyelitis viruses. Suckling and adult mice are unaffected as are guinea pigs, rabbits, and hamsters. 3. The donor of the virus was a febrile child with pleocytosis who developed specific neutralizing antibodies in high titer in convalescence. Similar antibodies have been detected in adult sera and in a pool of human gamma globulin.

---

1. Sabin, A. B., and Steigman, A. J., *Amer. J. Hyg.*, 1949, v49, 176.

2. Robbins, F. C., Weller, T. H., and Enders, J. F., *J. Immunol.*, 1952, v69, 673.

3. Wood, W., Franklin, A. E., Clark, E. M., Duncan, D., and Rhodes, A. J., PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 434.

4. Freund, J., *Ann. Rev. Microbiol.*, 1947, v1, 291.

5. Shwartzman, G., and Fisher, A., *J. Exp. Med.*, 1952, v95, 347.

6. Melnick, J. L., Ledinko, N., Kaplan, A. S., and Kraft, L. M., *J. Exp. Med.*, 1950, v91, 185.

7. Robbins, F. C., Enders, J. F., Weller, T. H., and Florentino, G. L., *Am. J. Hyg.*, 1951, v54, 286; also personal communication.

8. Contreras, G., Barnett, V. H., and Melnick, J. L., *J. Immunol.*, 1952, v69, 395.

9. Committee on Nomenclature of Nat. Found. for Infantile Paralysis, *Science*, 1948, v108, 701.

10. Huebner, R. J., Beeman, E. A., Cole, R. M., Beigelman, P. M., and Bell, J. A., *New Eng. J. Med.*, 1952, v247, 249, 285.

11. Melnick, J. L., and Agren, K., PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 621.

12. Unpublished observations.

Received April 16, 1953. P.S.E.B.M., 1953, v83.

Sublethal Total Body X-Radiation and Susceptibility of Mice to *Salmonella enteritidis* and *Escherichia coli*.* (20308)

I. L. SHECHMEISTER, L. J. PAULISSEN, AND M. FISHMAN.
(Introduced by C. G. Harford.)

From the Department of Microbiology, Washington University School of Medicine, St. Louis, Mo.

The results of a study dealing with the susceptibility of irradiated mice to infection with airborne *Streptococcus zooepidemicus* as a function of post-irradiation time(1) demonstrated a linear increase in susceptibility over the first 15 days, reaching a peak 5 times that of controls, followed by an exponential drop approaching the control level shortly after 30 days. Mice subjected to sublethal doses of radiation exhibited a time pattern of change in susceptibility to airborne infection with this organism, which was different from the time pattern reported for various other post-irradiation changes (peripheral blood counts, weight of certain organs, etc.). It was considered desirable to determine whether the established relationship between the two factors a) the degree to which radiation affects susceptibility to infection and b) the post-irradiation time interval required for such susceptibility to reach a maximum, would also apply to different microorganisms introduced by other than the natural airborne route. Certain results of such a study conducted with two contrasting organisms, the pathogenic *Salmonella enteritidis* and the usually innocuous *Escherichia coli,* are presented in this communication.

Materials and methods. Experimental design. Irradiated mice and control animals were inoculated intraperitoneally at the same time with graded concentrations of *S. enteritidis* at intervals over the 30-day period, and bacterial LD_{50} values for both the irradiated and control animals were calculated from the resulting mortality data and compared. In another series of experiments only a single bacterial concentration of *E. coli* (5×10^7 to 2×10^8 organisms) was introduced intraperitoneally at indicated time intervals to both normal and irradiated animals over the 60-day period following irradiation with different doses of X-rays. Bacterial LD_{50} value was not determined, since tenfold dilution of the administered bacterial suspension failed to kill any irradiated animals. *Microbial agents. S. enteritidis* 1891 (IX, XII . . . . g,m) was obtained from Dr. F. L. Adler, Harvard University School of Medicine, and was identified as culture 64 of the Agricultural Experimental Station of the University of Kentucky. In order to reduce variation, a lyophilized stock of the agent was prepared in individual ampoules. The entire content of a single ampoule was added to 10 ml of broth which, after static incubation for 10-12 hours at 37°C, constituted the first passage. The second passage was prepared by seeding 1 ml of the first passage per 50 ml of nutrient broth and incubating at 37°C for 18 hours. This second serial passage was used in all experiments. Prior to use, each culture was tested for purity and its concentration was determined. The *strain of E. coli* used in these experiments was isolated from the large intestine of a normal mouse. Lyophilized stock cultures and liquid cultures were prepared in the manner described for *S. enteritidis.* The *concentration* of bacterial cultures was determined at the time of inoculation of the organism by dropping a known quantity (about 0.02 ml) of bacterial suspension on E.M.B. agar (Difco) and counting colonies resulting after 18-24 hours of incubation at 37°C(2). *Experimental animals.* Six to 8 weeks old male and female Namru strain mice(3) from our own

---

* This investigation has been supported, in part, by Division of Biological and Medical Sciences, USN Radiological Defense Laboratory, San Francisco, Calif., and the U. S. Atomic Energy Commission.

The authors wish to express their appreciation to the following for their critical evaluation of the manuscript: Dr. V. P. Bond, U. S. N. Radiological Defense Laboratory, San Francisco, Calif.; Dr. G. Brecher, National Institutes of Health, Bethesda, Maryland; Cdr. E. P. Cronkite, MC-U.S.N., U.S.N. Medical Research Institute, Bethesda, Md.; and Dr. C. P. Miller, University of Chicago Medical School, Chicago, Ill.

TABLE I. Response of Mice to *S. enteritidis* as a Function of (a) Radiation Dose and (b) Post-Irradiation Time.

Total body radiation, r	Bacterial LD_{50} at stated post-irradiation periods						
	8 hr	1 day	5 days	12 days	15 days	20 days	30 days
350	6×10^1	8.8×10^1	3.3×10^1	3.6×10^2	6.7×10^2	3.7×10^4	3.8×10^5
250	5×10^2		4.7×10^4		1.1×10^6		4.5×10^5
50	8.4×10^5		4.5×10^5				
0	1×10^6	1.3×10^6	1.7×10^6	1.3×10^6	1.4×10^6	1×10^6	4.3×10^5

TABLE II. Total and Differential White Blood Cell Counts of Normal and Irradiated Mice.

Total body radiation, r	Post-irradiation period	WBC/mm^3, mean (10 mice) and stand. error of mean		
		Heterophils	Lymphocytes	Total
350	3 hr	1830 ± 140	2230 ± 240	4170 ± 280
	8 "	3920 ± 340	1180 ± 160	5200 ± 380
	1 day	1480 ± 80	700 ± 110	2200 ± 140
200	3 hr	1890 ± 250	2040 ± 140	3990 ± 330
	8 "	2880 ± 440	1250 ± 110	4230 ± 440
	2 days	1690 ± 170	1170 ± 100	2830 ± 160
0 (controls)		1170 ± 90	4140 ± 450	5960 ± 440
	Sham irradiated	1040 ± 90	7700 ± 610	8740 ± 670

breeding colony were used in the experiments. The colony was established in 1950 and, to date, has been free of any detectable infectious disease. The animals were irradiated by means of a Keleket deep therapy X-ray machine. The radiation factors were as follows: 200 KVP, 20 ma, filter 0.25 mm Cu and 1.0 mm Al, HVL 0.75 mm Cu, distance from target to skin 75 cm; output approximately 21 r per minute. All X-ray doses were measured in air. The results of the *X-ray mortality studies,* carried out in the manner previously described(1), confirmed the established observation that the maximal dose that could be given without causing deaths from radiation alone within 60 days was of the order of 350 r. Groups of animals were exposed to doses of X-ray ranging from a) 50 to 350 r, prior to challenge with *S. enteritidis,* and b) 50 to 450 r, prior to inoculation with *E. coli.* Five to six animals were used per dilution in determination of LD_{50} values for *S. enteritidis.* However, when the mortality end point for intraperitoneal inoculation of *E. coli* was investigated, at least 10 and usually 20-30 or more animals were used for a dose of X-ray. All the LD_{50} values were calculated according to the method of Reed and Muench(4). Representative number of mice dying after challenge with *S. enteritidis* were autopsied and cultured for this organism. Total and differential white blood cell counts were made on tail-vein blood of groups of 10-12 animals: a) Exposed to 350 r or 250 r at different post-irradiation periods; b) sham-irradiated mice, and c) normal controls.

Results. Exposure to 350 r increased susceptibility of mice to infection with *S. enteritidis* (Table I). The results were most marked 8 hours to 5 days after irradiation, when bacterial LD_{50} was of the order of 30-100 organisms for the irradiated as opposed to the LD_{50} value of about 1.0 x 10^6 to 1.7 x 10^6 organisms for the normal animals. In the course of time this effect gradually decreased until, some 30 days after irradiation, the susceptibility was the same as for non-irradiated animals. Animals exposed to 250 r also showed a marked increase in susceptibility to this agent when challenged 8 hours after irradiation. However, a pronounced rise in the bacterial LD_{50}, evident within 5 days, approached that for normal mice in 15 days. Exposure to 50 r total body radiation prior to inoculation did not seem to influence the susceptibility to this infection. Post-mortem cultures of representative number of mice from each of the groups indicated in Table I revealed the presence of Salmonella in the spleens of all of the tested animals. Although no significant difference in total leucocyte count was noted within 8 hours after exposure to 250 r or to 350 r, a significant lymphopenia and a concomitant granulocytosis were present at this time (Table II).

Since bacteremia primarily due to *E. coli* was positively correlated with mortality after exposure to lethal doses of X-rays(5), it appeared desirable to evaluate the effect of mid-lethal and sublethal radiation on the susceptibility of mice to this agent administered intraperitoneally, at different times after irradiation. The results of representative experiments are indicated in Table III. Of the several groups of animals used as controls, it was only in the 30-day post-irradiation group that some mice died following bacterial inoculation. As is seen from Table III, exposure of mice to 450 r and to 350 r total body radiation resulted in death of a certain number of animals. This was considered in calculation of the per cent mortality of groups of animals subjected to the respective dose of radiation. Practically all deaths from radiation alone occurred within 8-20 days following irradiation; however, the average survival period of all irradiated animals inoculated with *E. coli* was of the order of 2 to 4 days, occasionally 6 days.

Increase in susceptibility to *E. coli* was observed as early as one day after irradiation. In the group exposed to 450 r there was a gradual decline in susceptibility until, when tested 60 days after irradiation, mortality rate of 37% was observed. Previous exposure to 350 r resulted in a marked susceptibility to infection, demonstrated within one day and sustained through 11 days, and declining to 20% 24 days after irradiation. Exposure to 200 r brought about an initial increase in the susceptibility, which returned to normal in animals tested 24-30 days after irradiation. After exposure to 50 r, increase in susceptibility to *E. coli* was observed only when challenged 11 days after irradiation.

Discussion. Granulocytosis and lymphopenia were observed in mice 3-8 hours after exposure to 500 r whole body radiation(6). In our study granulocytosis was also observed in mice 8 hours after exposure to 250 r or

TABLE III. Response of Mice to *E. coli* as Function of (a) Radiation Dose and (b) Post-Irradiation Time.

Post-irradiation period, days	450 r							350 r					200 r†			50 r†		
	A		B (controls)					A		B‡ (controls)								
	Inoculated Dead/total	%	Radiation *Dead/total	%	Bacterial Dead/total	%	A–B, final % mortality	Inoculated Dead/total	%	Radiation *Dead/total	%	A–B, final % mortality	Inoculated Dead/total	%	Final % mortality‡	Inoculated Dead/total	%	Final % mortality‡
1	15/15	100	9/341	3		(0)§	97	10/10	100	†		100	7/10	70	70	2/10	20	20
4	19/20	95	113/450	25	0/10	0	70	19/20	95	1/117	1	94	5/10	50	50	0/10	0	0
7	15/15	100	125/446	28		(0)§	72	10/10	100	7/117	6	94	6/10	60	60	1/10	10	10
11					0/10	0		9/11	82	16/116	14	68	20/25	80	80	14/23	61	61
24	5/9	56	5/222	2		(0)§	54	4/19	21	1/91	1	20	3/18	17	17	4/23	17	17
30	26/28	93	4/213	2	17/44	39	52	22/41	54	†		15	15/37	40	1	9/40	23	0
60	11/25	44	†		1/14	7	37	3/14	21	†		14	0/22	0	0	1/27	4	0

* Cumulative results of several experiments.

† No deaths due to radiation observed.

‡ Same bacterial controls as used for 450 r group.

§ Evidence from overall experience supports the conclusion that, in general, occurrence of deaths in bacterial controls is very unlikely, and that the 39% mortality for the 30-day period is exceptional.

to 350 r. It is, therefore, apparent that the increased susceptibility to the experimental (intraperitoneal) infection at this post-irradiation period cannot be accounted for by granulocytopenia at the time of injection. The pattern of total, and differential white blood cell counts for certain other periods following irradiation also indicates no consistent correlation between granulocyte count and susceptibility to infection. The present data may possibly be explained either by a damage to some system involved in the defense against this experimental infection other than that provided by the granulocytes, or by an alteration in functional ability of granulocytes. However, no implication regarding the possible role of granulocytopenia in natural infections of irradiated animals is intended by this statement.

Inoculation of *E. coli* at different times after exposure to 50 r reveals an increase in susceptibility to this microorganism only in the group of mice that were challenged 11 days after irradiation. This peak is of interest in the light of the high mortality and bacteremia reported initially at that particular post-irradiation period by Miller *et al.*(7) in mice subjected to 450 r total body radiation, an observation frequently confirmed in this laboratory. The 11-day peak in increased susceptibility to infection was only suggested after exposure to 200 r, but was not apparent at higher doses, probably because it formed a part of the "post-irradiation susceptibility range" initially evident one day after irradiation.

Exposure to sublethal radiation increases the susceptibility of certain animals to experimental infection with bacterial(8), fungal(9), viral(10) and rickettsial agents(11,12), as well as bacterial toxins(13). The increased susceptibility to infection by mice challenged with airborne *Str. zooepidemicus* was shown to be a function of the post-irradiation time, with the peak slowly reaching a maximum 15 days after irradiation. However, a very pronounced increase in susceptibility to the enteric organisms was noted 8-24 hours after irradiation. While inhalation of *Str. zooepidemicus* stimulated a natural portal of entry of the organisms, intraperitoneal administration of *S. enteritidis* and *E. coli* represented an artificial situation with bacteria by-passing the "primary line of defense", exemplified in the case of *Str. zooepidemicus* by the intact respiratory mucosa. Apparently radiation rapidly brought about alteration of "the secondary defense mechanisms", to the extent that the extremely pronounced increase in susceptibility to *S. enteritidis* was observed 8 hours after irradiation, and to *E. coli* in one day.

The importance of the time of challenge in relation to radiation (*i.e.*, post-irradiation period) very evident here, has also been pointed up by variation in susceptibility of a) mice exposed to a single total-body irradiation in the LD_{50} range followed by intramuscular administration of a beta hemolytic streptococcus at different post-irradiation periods(13), and b) sublethally irradiated mice to toxin of *Clostridium septicum*(14) and influenza virus(15). Similarly, the importance of the route of infection was also demonstrated in experiments with *Corynebacterium pseudotuberculosis murium*(16).

Summary. 1. Mice were challenged intraperitoneally with *S. enteritidis* over a 30-day period following exposure to 50-350 r whole body X-radiation. A very marked increase in susceptibility to infection was observed as early as 8 hours after irradiation with 250-350 r, but not after 50 r. This increase in susceptibility cannot be explained by granulocytopenia at the time of injection, since granulocytosis was observed in peripheral blood of mice at this post-irradiation period. 2. A very marked increase in susceptibility to intraperitoneal inoculation with *E. coli* was observed one day following irradiation with 200-450 r. This gradually declined but was still evident 60 days after exposure to 450 r, and 11 days after exposure to 200-350 r. After exposure to 50 r, the single peak of susceptibility to *E. coli* occurred in 11 days. 3. The relationship of the route of inoculation and the post-irradiation time to the degree of susceptibility to infection is discussed.

The authors gratefully acknowledge the cooperation of Dr. Hugh M. Wilson of Mallinckrodt Institute of Radiology in making available the facilities for irradiation of animals. It is a pleasure to acknowledge the capable technical assistance of Miss Rita Yunker.

---

1. Shechmeister, I. L., Bond, V. P., and Swift, M. N., *J. Immunol.*, 1952, v68, 87.

2. Miles, A. A., and Misra, S. S., *J. Hyg.*, 1938, v38, 732.

3. Garber, E. D., and Hauth, F. C., *J. Hered.*, 1950, v41, 122.

4. Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, v27, 493.

5. Miller, C. P., Hammond, C. W., and Tompkins, M., *Science*, 1950, v111, 540.

6. Widmann, H., and Ludwig, R., *Strahlenther.*, 1952, v89, 243.

7. Miller, G. P., Hammond, C. W., and Tompkins, M., *J. Lab. Clin. Med.*, 1951, v38, 331.

8. Shechmeister, I. L., and Bond, V. P., PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 77.

9. Syverton, J. T., Werder, A. A., Friedman, J., Roth, F. J., Jr., Graham, A. B., and Mira, O. J., PROC. SOC. EXP. BIOL. AND MED., 1952, v80, 123.

10. deGara, P. F., and Furth, J., *J. Immunol.*, 1945, v50, 255.

11. Zinsser, H., and Castaneda, M. R., PROC. SOC. EXP. BIOL. AND MED., 1932, v29, 840.

12. Greiff, D., Chiga, M., Blumenthal, H. T., and Pinkerton, J., *J. Exp. Med.*, 1953, v97, 139.

13. Adler, F. L., and Shechmeister, I. L., PROC. SOC. EXP. BIOL. AND MED., 1952, v80, 660.

14. Kaplan, H. S., Speck, R. S., and Jawetz, E., *J. Lab. Clin. Med.*, 1952, v40, 682.

15. Shechmeister, I. L., Unpublished observations.

16. Shechmeister, I. L., and Adler, F. L., *J. Infect. Dis.*, 1953, v92.

Received April 17, 1953. P.S.E.B.M., 1953, v83.

Aortic Albuminoid Titration and Swelling Curves from Persons Differing in Age and Degree of Atherosclerosis. (20309)

HOWARD MILLER AND DOROTHY M. KRAEMER.

From the Department of Anatomy, State University Medical Center, College of Medicine, Brooklyn.

The present investigation is concerned with one phase of a study on the chemical changes associated with atherosclerosis and increasing age of the human aorta. Titration and swelling curves of the human aortic albuminoid (collagen, reticulin, and elastin) have been studied with reference to possible changes in protein constitution associated with atherosclerotic processes. Previously, the existence, in the human aortic albuminoid, of a carbohydrate fraction not quantitatively altered by pathological or aging changes has been demonstrated(1). However, albuminoids from aged and atherosclerotic aortae have been shown to combine with larger quantities of hydrochloric acid than do relatively normal or younger samples, although combination with sodium hydroxide remained constant in all cases(2). The mean percentage of elastin in this aortic albuminoid showed no significant change with age or atheromatous condition. However, a statistically significant increase in albuminoid elastin was apparent in samples showing visible calcification(3).

Materials and methods. The albuminoid samples were prepared from 23 human aortae by methods previously described(2). Following desiccation, each sample was ground in a Wiley mill (40 mesh) and replaced in the desiccator for a minimum of 24 hours. The volume swelling and titration curves were determined by the method of Highberger(4), which was slightly modified according to the special needs of the present problem. Eleven 100 mg samples from each aorta were weighed out on an analytical balance and placed in an equal number of graduated 15 ml centrifuge tubes. Ten ml of the appropriate standard solution was added to each tube and the contents shaken. Five samples were incubated in hydrochloric acid standards (0.0005N-0.05N), 5 in sodium hydroxide standards (0.00055N-0.055N), and one in distilled water. All samples were stoppered and incubated for 48 hours at room temperature. Following the incubation period, the tubes were centrifuged for 10 minutes at about 400 g. The volume of solid was estimated imme-

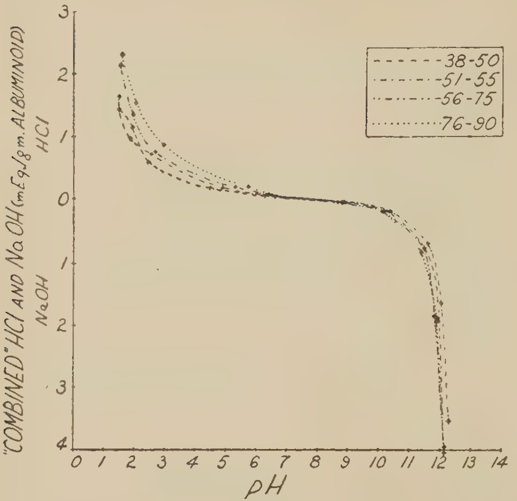


FIG. 1. Effect of age on titration curve of human aortic albuminoid (the 4 age groups contained, in chronological order, 4, 6, 6, and 6 samples).

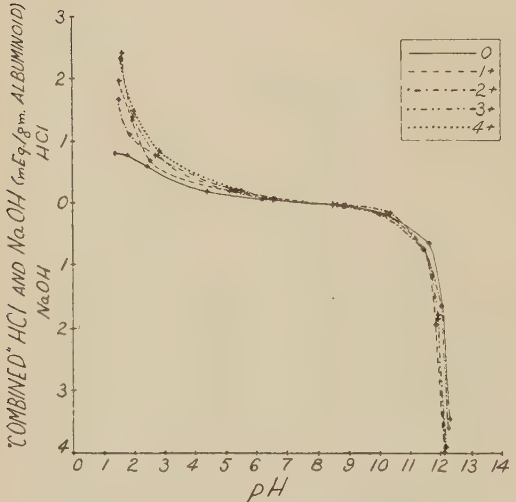


FIG. 2. Effect of pathological changes on titration curve of human aortic albuminoid. On the basis of gross observations, 0 = normal, 1+ = few atheromatous plaques, 2+ = moderate atheromatous plaques, 3+ = atheromatous plaques and few calcified plaques, and 4+ = many plaques, atheromatous and calcified. The groups contain 3, 6, 3, 6, and 5 samples, respectively.

diately following centrifugation. The pH of each supernatant solution was then determined with a Beckman pH Meter using the glass electrode. The alkaline pH readings were corrected for sodium ion errors and the number of milliequivalents of acid and base "combined" by each aliquot calculated from the above data.

Results. The data obtained from the aortae were arbitrarily subdivided into 4 age groups (Fig. 1, 3) and again into 5 groups graded according to the extent of atherosclerotic change (Fig. 2, 4). The latter were defined on the basis of gross observations. The average results within each age and pathological group were calculated and titration

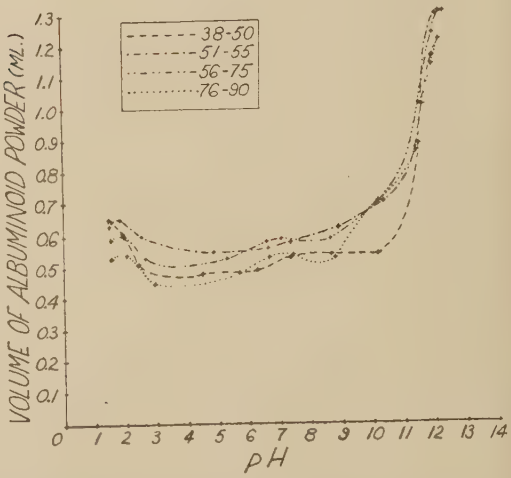


FIG. 3. Effect of age on swelling curve of human aortic albuminoid (the 4 age groups contained, in chronological order, 4, 6, 6, and 6 samples).

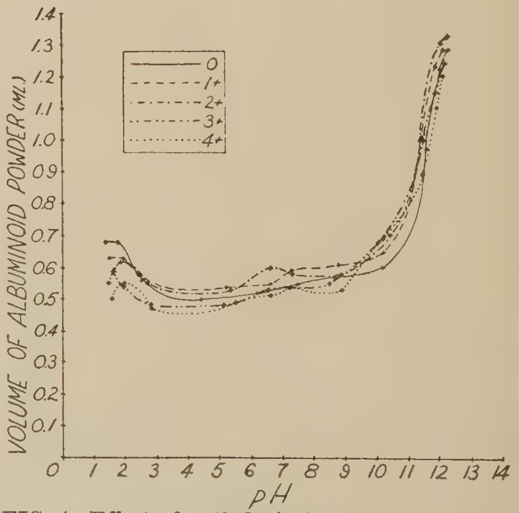


FIG. 4. Effect of pathological changes on swelling curve of human aortic albuminoid. On the basis of gross observations, 0 = normal, 1+ = few atheromatous plaques, 2+ = moderate atheromatous plaques, 3+ = atheromatous plaques and few calcified plaques, and 4+ = many plaques, atheromatous and calcified. The groups contain 3, 6, 3, 6, and 5 samples, respectively.

TABLE I. Swelling and Titration Properties of the Albuminoid of 23 Human Aortae.

Incubating solution	Mean vol ± stand. dev.	Mean pH ± stand. dev.	Mean combined HCl or NaOH (mEq/g) ± stand. dev.
N			
.05 HCl	.59 ± .09	1.54 ± .09	1.97 ± .70
.025 ”	.59 ± .14	1.94 ± .17	1.26 ± .40
.01 ”	.52 ± .11	2.68 ± .25	.75 ± .09
.002 ”	.51 ± .05	5.19 ± .71	.20 ± .00
.0005 ”	.54 ± .06	6.57 ± .21	.05 ± .00
Distilled H_2O	.56 ± .09	7.31 ± .52	—
.00055 NaOH	.57 ± .06	8.75 ± .67	.05 ± .00
.0022 ”	.67 ± .13	10.21 ± .67	.20 ± .02
.011 ”	.97 ± .19	11.50 ± .64	.76 ± .29
.0275 ”	1.19 ± .18	11.95 ± .67	1.81 ± .27
.055 ”	1.26 ± .09	12.19 ± .67	3.85 ± .47

and swelling curves plotted using these mean values (Fig. 1-4). The combined data were also computed (Table I). The titration curve (Fig. 1, 2) for the human aortic albuminoid was characterized by minimal acid and base combining power in the approximate pH range of 3-11. Below, and, especially, above this range, relatively large quantities of acid and base were combined. No marked effects of aging or pathologic change were observed, except for the apparent trend towards increased combination with hydrochloric acid with increasing age and pathology, consistent with previous findings(2).

The albuminoid swelling properties with respect to age (Fig. 3) and pathologic change (Fig. 4), were typified by pronounced increases in volume in the higher pH range (10-12). Again, no major changes resulting from increasing age or increasing pathologic change could be inferred from either curve.

Discussion. The problem of hydrolysis presents a complicating factor in attempting to interpret the observed data. It has been shown in similar experiments that the amount of protein nitrogen dissolving from steerhide collagen is greatest in the more basic solutions(4). This peak value, however, represents only 6.4% hydrolysis when calculated as an equivalent weight of collagen.

It is of interest to compare the present results with those obtained from steerhide collagen(4). The characteristic broadening of the isoelectric point into an isoelectric range observed in fibrous proteins can be shown in both cases. The results differ mainly in that steerhide collagen combines with more acid and less base at the extreme pH ranges than does the human aortic albuminoid. Likewise, steerhide collagen swells maximally at about pH 2.5, while aortic albuminoid reaches its swelling peak at approximately pH 12. In both cases, however, the similarity between titration and swelling curves of the same albuminoid sample is evident.

Though all the curves plotted are not everywhere identical in respect to aging and pathological changes, no consistent differences comparable to the marked changes observable in this tissue upon microscopic or visual examination can be found. The possible exception to this statement concerns those changes in acid-combining properties previously noted (2), which may be due to differences in ash content of the albuminoids. Though the conditions of the two sets of experiments were somewhat different, it seems of value to compare the results of the present investigation with those concerning the combination of hydrochloric acid and sodium hydroxide with the aortic albuminoid(2). The maximum amount of acid combined in the latter data was about 2.3 milliequivalents per g of albuminoid. The present work is essentially in agreement with that figure (1.97 mEq/g). On the other hand, 3.85 milliequivalents of base were bound maximally by the albuminoid in the present series, while the earlier data show a peak combination with only slightly above 0.8 milliequivalent per g of albuminoid.

This discrepancy is probably associated with the greater incubation time used in the recent experiments. Thus, a substantially larger amount of hydrolysis of the proteins incubated in sodium hydroxide may have taken place with prolonged standing.

Summary. 1. Titration and swelling curves of the human aortic albuminoid were determined and analyzed with respect to possible effects of increasing age and atherosclerosis. Except for previously determined differences in acid-binding, no marked changes were observed. 2. Titration curves were characterized by extension of the isoelectric point into an isoelectric range between approximately pH 5 and 10. Maximal swelling was observed at approximately pH 12.

Authors wish to express their appreciation to Miss Grace O'Brien and Miss Jean Wright for their technical assistance.

---

1. Miller, H., Haft, H., and Kraemer, D. M., PROC. SOC. EXP. BIOL. AND MED., 1952, v79, 411.

2. Miller, H., and Kraemer, D. M., PROC. SOC. EXP. BIOL. AND MED., 1952, v80, 243.

3. Kraemer, D. M., and Miller, H., *Arch. Path.*, 1953, v55, 70.

4. Highberger, J. H., *J. Am. Leather Chem. Assn.*, 1936, v31, 345.

Received April 21, 1953. P.S.E.B.M., 1953, v83.

Cholesterol and Cholate Concentration of Perfusate and Bile of Isolated Liver Preparation.* (20310)

EDWARD D. ROBBINS, STANLEY BURTON, JON GOERKE, AND MEYER FRIEDMAN.
(With the technical assistance of Tadashi Ishida and Charles Calvert.)
From the Harold Brunn Institute, Mount Zion Hospital, San Francisco, Calif.

A perfusion apparatus has been described by Brauer, Pessatti, and Pizzolato(1) in which the isolated rat liver can be maintained in a viable state for many hours, and its functional capacity demonstrated by the continuous formation of bile. The present report describes the cholesterol and cholate content of the perfusate, and of the bile, in 12 perfusion experiments on the isolated rat liver.

Methods. Young, male, non-fasted, Long-Evans rats in a weight range of 220-350 g were utilized. Ether inhalation anesthesia was employed in all instances (in some cases, intraperitoneal nembutal (12 mg) was given initially). After opening the abdomen, 1.0 mg of heparin was injected into the inferior vena cava. The bile duct, portal vein, and thoracic vena cava were cannulated in turn with polyethylene catheters. The liver was excised and placed in the perfusion apparatus, with perfusate entering the portal vein catheter under a constant, non-pulsatile pressure of 21.5 cm. Clean, but not sterile, surgical technic was employed. The operative time during which circulation through the liver was interrupted, *i.e.*, from ligation of the portal vein until placement of the excised liver in the perfusion apparatus, was under 10 minutes in all cases, and averaged 5 minutes. The perfusate consisted of one part of heparinized whole rat blood diluted with 3 parts of salt solution, prepared according to Brauer(1). Dextrose sufficient to achieve a concentration of 80 mg % was added to the complete perfusate. In all but the first 4 experiments, an additional 4.0 cc of 10 "essential" amino acids was added, as prepared by White(2). In the last 4 experiments 4.0 cc of 9 of the B vitamin group, and 4.0 cc containing carotene, Vit. A, ascorbic acid, glutathione, and cysteine were added, also as prepared by White(2). An infusion containing the same salt and albumin† concentrations as the perfusate with the addi-

---

* Aided by grants from the California Charities Foundation, the Monterey Heart Assn., the San Francisco Heart Assn., and the U. S. Public Health Service.

† The authors wish to thank Armour and Co. for supplying the bovine plasma albumin used in these experiments.

TABLE I. Cholesterol and Cholate Concentration of Perfusate and Bile of Isolated Liver Preparation.

	Duration of perfusion		Perfusate					Bile		
				Cholesterol		Cholate				
	Hr	Min.	Flow* (cc/min.)	Initial (mg %)	Final (mg %)	Initial (mg %)	Final (mg %)	Flow (cc/hr)	Cholesterol (mg %)	Cholate (mg %)
Avg:	7	25	49	13.8	18.0	1.2	1.4	.24	6.3	117
Range:	3 11	40 to 10	7–95	8.3–17	8.3–30	.0–2.6	.0–3.0	.10–.61	3.5–11.6	59–240
S.E. mean†:			4.6	1.6	2.0	.42	.32	.04	.86	17
No. of observations:	12		12	5	11	5	11	12	8	10

* Avg flow values calculated as mean of values observed at beginning, middle, and end of perfusion period.

† Stand. error of mean calculated by formula S.E. = $\sqrt{\dfrac{\Sigma x^2}{N^2}}$.

tion of .5% of dextrose, was injected at a rate of .29 ml/hour. To control bacterial growth, terramycin was added, first to the infusion, later directly to the perfusate in a concentration of 0.01 mg/ml. Samples of perfusate and bile were removed for analysis of cholesterol and cholate according to technics previously described from this laboratory(3,4). The average initial volume of the perfusate was 95 ml. The perfusate gave an hematocrit reading of about 10%; the pH varied from 7.1 to 7.3; the proteins were 2.5 g %. Oxygen saturation was maintained at 92%, temperature at 37°C.

Results. The following data were compiled from 12 perfusion experiments.

I. *Perfusate.* The average flow maintained through the hepatic circulation during the period of perfusion was 49 cc per minute. The rate of flow through the liver generally increased, during the first half-hour, from a minimum to the average value and continued unabated at this rate until the experiment was terminated at the cessation of bile flow. The cholesterol content of the perfusate at the start of the perfusion averaged 13.8 mg % (range: 8.3-17 mg %). At the end of the perfusion, the cholesterol content averaged 18.0 mg % (range: 8.3-30 mg %). The plasma cholesterol of intact rats averages 48 mg %) (range: 32-76 mg %(7)). The difference between initial and final perfusate cholesterol levels is within three standard errors of the mean and is not considered significant. The initial level of perfusate cholate averaged 1.2 mg % (range: 0.0-2.6 mg %). The final concentration averaged 1.4 mg % (range: 0.0-3.0 mg %). The average cholate concentration of intact rats recently obtained in this laboratory is 3.5 mg % (range: 0.5-4.5 mg %(7)).

II. *Bile.* The average bile flow was 0.24 cc per hour. This is 41% of the average rate of 0.59 ml/hour secreted by the intact animal (5). The total amount of bile collected from each preparation averaged 2.0 cc (range: 0.68-4.9 cc). The average cholesterol content of the bile was 6.3 mg % (range: 3.5 mg % to 11.6 mg %). The cholesterol concentration of bile obtained from intact rats averages 55 mg % (range: 42-69 mg %). The amount of cholesterol secreted per hour was 0.0183 mg. This is 22.5% of the 0.075 mg per hour secreted by the intact rat(7). The average cholate level in the bile was 117 mg % (range: 59-240 mg %). In the intact rat biliary cholate concentration averages 184 mg % (range: 98-290 mg %(7)).

Discussion. The above studies demonstrate that the isolated perfused rat liver not only can be maintained in a viable state over a period of many hours as demonstrated by the continuous formation of bile, but will continue to secrete cholesterol and cholate into the bile. Since biliary cholesterol has been shown to be an index of hepatic synthesis of cholesterol(5) and biliary cholate to be the major excretion product of the hepatic destruction of cholesterol(6), these data demonstrate the integrity of these vital processes of cholesterol synthesis and degradation in the isolated perfused rat liver.

Summary. When the isolated rat liver was perfused with a solution containing whole rat's blood diluted 1:3 with a solution of electrolytes, crystalline albumin, amino acids, and vitamins, bile was continuously formed over a period of 2 to 11 hours. The initial perfusate level of cholesterol averaged 13.8 mg %, and the initial perfusate level of cholate averaged 1.2 mg %. The final perfusate levels of cholesterol averaged 18.0 mg %, and of cholate 1.4 mg %. The differences between initial and final concentrations are not significant for these data. The bile contained an average of 6.3 mg % of cholesterol and 117 mg % of cholate. These data demonstrate that the isolated perfused rat liver maintains its ability to synthesize and degrade cholesterol.

The authors wish to express their thanks and appreciation to Ralph W. Brauer, for his assistance and advice.

---

1. Brauer, R. W., Pessotti, R. L., and Pizzolato, P., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 174.
2. White, P. R., *Growth,* 1946, v10, 231.
3. Byers, S. O., Friedman, M., and Michaelis, F., *J. Biol. Chem.,* 1950, v184, 71.
4. Friedman, M., Byers, S. O., and Michaelis, F., *Am. J. Physiol.,* 1951, v164, 786.
5. Byers, S. O., and Friedman, M., *Am. J. Physiol.,* 1952, v168, 297.
6. Friedman, M., Byers, S. O., and Gunning, B., *Am. J. Physiol.,* 1953, v172, 309.
7. Friedman, M., and Byers, S. O., *J. Exp. Med.,* 1953, v97, 117.

Received May 4, 1953. P.S.E.B.M., 1953, v83.

Mechanical Fragility of Erythrocytes from Leukemic Patients.* (20311)

ROBERT F. SCHILLING AND EDITH R. EISENHAUER.
From the Department of Medicine, University of Wisconsin Medical School.

The theory that an excessive rate of hemolysis might be a causative factor in the anemia of leukemia is not new(1). Jaffe's(2) observation of hemosiderosis in the organs of leukemic patients who had not been transfused led him to emphasize the probability of an increased red cell destruction in this disease. In the past few years R. Berlin(3) and Aas(4) and Brown *et al.*(5) have reported that donor erythrocytes have a sub-normal life span in leukemic patients, even when the latter failed to exhibit the usual signs of hemolytic anemia. Ross and his colleagues(6) have demonstrated increased fecal urobilinogen and shortened red cell life in patients with leukemia. The red cell survival was estimated in the latter report by radioactive iron tagging of the patient's erythrocytes, while in the study by R. Berlin the survival of transfused cells was studied by the Ashby technic. N. I. Berlin *et al.*(7) have demonstrated a shortened red cell life in some leukemic patients by glycine C^{14} tagging of their red cells. Watson and Hagen(8) have reported some instances of frank hemolytic anemia in patients with leukemia.

The mechanical fragility (M.F.) of the erythrocytes is increased in several varieties of hemolytic anemia, *e.g.*, congenital spherocytosis(9), thermal burns(9), some cases of acquired hemolytic anemia(10), methemoglobinemia with hemolytic anemia(11), malaria, and the sickled cells of sickelemia(9). A similar increase in the M.F. of the erythrocytes from leukemic patients is reported in this paper.

Methods. One-half ml of oxalated(12) venous blood with the hematocrit adjusted to 35% by adding or removing plasma was rotated at 44 RPM at room temperature for one hour in a 50 ml Erlenmeyer flask containing 10 glass beads 4 mm in diameter. The radius of the circle through which the flasks turned was 8.7 cm. The apparatus and method are similar to that described by Shen, Fleming, and Castle(8). The hemoglobin appearing in the plasma of the rotated sample was measured in an Evelyn colorimeter and expressed as a per cent of the total hemoglobin present in ½ ml of blood. Blood from 43 leukemic patients was studied. The "control" values were determined on 43 persons, either healthy people or hospitalized patients with illnesses considered unrelated to the present study. The controls were selected to match the patients in age. In the leukemic group there were 16 patients with chronic lymphocytic leukemia, 13 with chronic granulocytic and 14 with acute leukemia. Fecal urobilinogen values were determined by Watson's technic (13), utilizing a 4-day stool collection.

---

* Supported in part by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

TABLE I. Mechanical Fragility of Erythrocytes in Leukemia, in 43 Cases.

Age	Sex	Diagnosis	WBC × 10^3	HGB, g/100 ml	M.F. %
63	♂	AL(?)	5.0	7.3	2.2
63	♂	CGL	30.4	9.9	2.6
65	♂	"	30.7	10.0	2.7
2	♂	AL	96.2	6.8	3.0
85	♀	CLL	49.6	11.9	3.1
46	♀	"	9.4	11.5	3.4
37	♂	CGL	18.1	16.5	3.5
65	♂	CLL	23.6	14.0	3.7
67	♂	"	24.0	15.4	4.1
69	♂	"	70.5	14.0	4.1
68	♂	"	66.5	12.3	4.1
40	♀	AL	5.9	10.8	4.4
19	♂	"	14.3	9.5	4.7
64	♂	CGL	28.3	11.5	4.8
2	♂	AL	7.4	10.2	5.0
43	♀	CGL	809.1	10.0	5.2
18	♀	AL	7.0	12.0	5.7
22	♀	"	64.3	14.0	5.8
55	♀	CLL	20.5	11.3	5.9
53	♂	AL	220.1	7.5	5.9
57	♀	CLL	103.5	12.7	5.9
14	♂	"	113.0	6.8	5.9
50	♂	"	68.2	16.0	6.1
68	♀	CGL	119.5	11.1	6.2
62	♀	"	297.0	10.5	6.2
61	♂	CLL	282.1	7.1	7.0
59	♂	"	25.1	12.2	7.1
65	♀	CGL	257.0	11.8	7.7
45	♀	"	79.6	13.7	7.8
49	♂	CLL	221.5	4.8	7.9
75	♂	AL	84.0	10.6	7.9
59	♀	"	4.9	11.8	8.0
23	♀	CGL	78.4	8.8	8.3
62	♂	AL	1.1	6.4	8.9
50	♀	CGL	72.5	13.2	9.1
66	♂	"	141.8	6.7	9.1
64	♀	AL	5.9	9.1	9.5
3	♂	"	6.9	6.7	9.6
39	♀	"	33.5	7.6	9.8
59*	♂	CLL	55.9	4.8	10.0
66	♂	"	59.5	11.0	10.8
59*	♂	"	11.3	3.7	11.9
30	♂	CGL	184.5	12.5	13.0
		Avg		10.3	6.46

* Positive Coomb's test.

TABLE II. Summary of Statistical Analysis of Mechanical Fragility of Erythrocytes.*

	Avg M.F. %	Range	S.D.†
43 leukemic patients	6.46	2.2–13.0	± 6.91
(26 male)	6.4		
(17 female)	6.6		
43 controls	4.38	2.1– 6.4	± 1.26
(19 male)	4.6		
(24 female)	4.2		

t = 4.698—significant at the 1% level of significance.

* The University of Wisconsin Computing Service obligingly analyzed the data summarized in Table II.

† S.D. = Stand. deviation.

Results and discussion. Tables I and II show the pertinent data. The average M.F. for the control group was 4.4% and for the leukemic group it was 6.5%. The average for the 14 patients with acute leukemia was 6.5%, for chronic granulocytic 6.6%, and for chronic lymphocytic 6.1%.

The Coomb's test was positive in 2 of 10 bloods studied from patients with chronic lymphocytic leukemia. The M.F. of these 2 bloods was 10% and 11.9%, and the patients excreted 663 mg and 348 mg of fecal urobilinogen per day.

The average hemoglobin level for the 43 leukemic patients was 10.3 g/100 ml. The 12 having an M.F. of at least 8% had an average hemoglobin of 8.5 g/100 ml, while those with an M.F. of less than 8% had an average hemoglobin level of 11.1 g/100 ml. Interpretation of these data is difficult because transfusions had been given to some patients before the M.F. was determined, but the average M.F. of blood from 20 patients who had received no transfusions was 6.2%, not significantly lower than the average of 6.5% for the total group of 43.

Eighteen of the 43 leukemic bloods (42%) had an M.F. value which exceeded the highest value in the control group.

The M.F. of erythrocytes from one woman with chronic granulocytic leukemia was determined 5 times over an interval of 19 months with the following results:

Date	M.F. %	Hematocrit
7/51	6.2	35
10/51	8.3	30
6/52	3.9	36
8/52	8.5	31
3/53	1.9	49

In March, 1953, at the time of the most recent M.F. determination the patient was enjoying a remission following therapy.

An increased M.F. is common to several varieties of hemolytic anemia, and the finding of an increased M.F. of the red cells from leukemic patients is consistent with previous studies of a different nature indicating an increased rate of red cell destruction as a contributory factor in the anemia of leukemic patients.

Summary and conclusions. The average mechanical fragility of the erythrocytes from 43 leukemic patients was 6.5%, while that of the control group was 4.4%. Statistical analysis of the reported data indicates that this difference is not due to chance.

It is a pleasure to acknowledge the help of Miss Margaret Thompson with some of these determinations.

1. Berlin, R., *Acta Med. Scan.*, 1951, Suppl. 252.
2. Jaffé, R. H., *Arch. of Path.*, 1935, v20, 725.
3. Berlin, R., *Acta. Med. Scan.*, 1951, v139, 331.
4. Aas, quoted by R. Berlin(3).
5. Brown, M. G., Elliott, M. S., Young, W. A., *J. Clin. Invest.*, 1951, v30, 130.
6. Ross, J. F., Crockett, C. L., Emerson, C. P., *J. Clin. Invest.*, 1951, v30, 668.
7. Berlin, N. I., Lawrence, J. H., and Lee, H. C., *Science*, 1951, v114, 385.
8. Watson, C. J., Hagen, P. S., *Proc. of the Third Int. Congress of the Int. Soc. of Hem.*, 1950, 95.
9. Shen, S. C., Castle, W. B., Fleming, E. M., *Science*, 1944, v100, 387.
10. Wagley, P. F., Shen, S. C., Gardner, F. H., and Castle, W. B., *J. Lab. Clin. Med.*, 1948, v33, 1197.
11. Schilling, R. F., unpublished observations.
12. Wintrobe, M. M., *Clinical Hematology*, 3rd Edition, Lea and Febiger, Philadelphia, 1951.
13. Watson, C. J., *Am. J. Clin. Path.*, 1936, v6, 458.

Received May 4, 1953. P.S.E.B.M., 1953, v83.

Evidence for Lipolytic Action by Human Plasma Obtained after Intravenous Administration of Heparin.* (20312)

B. SHORE, A. V. NICHOLS, AND N. K. FREEMAN. (Introduced by J. H. Lawrence.)

From the Donner Laboratory of Medical Physics, and the Radiation Laboratory, University of California, Berkeley.

The action of heparin *in vivo* has become a focal point in the study of serum lipoproteins and their relationship to the development of atherosclerosis. It was observed by Hahn(1) that intravenous injection of heparin could effectively clear lipemic blood in humans. More recently, it has been shown by Graham *et al.*(2) in this laboratory, using ultracentrifugal technics, that drastic alterations in the serum lipoprotein distribution could also be produced by heparin *in vivo*. These effects have not been observed when heparin was allowed to react with serum *in vitro;* but plasma obtained from a human subject 15-30 minutes after the administration of heparin can evidently interact with certain classes of lipoproteins on incubation to cause changes in their ultracentrifugal pattern and other observable effects. For brevity, such plasma will be referred to as post-heparin plasma. Attempts to isolate or concentrate an active component by chemical fractionation of post-heparin plasma have been reported by other workers(3), and ultracentrifugal fractionation is being carried out in this laboratory toward a similar goal. The most pronounced transformations that occur as a result of reaction with post-heparin plasma involve glyceride-containing lipoproteins of the lower density classes. These classes tend to diminish or disappear, whereas the lipoproteins of higher density may increase in quantity. Another type of effect, which can be correlated with the ability of such plasma to cause lipoprotein changes, is the reduction in turbidity of a solution of egg yolk lipoprotein when incubated with the plasma under specified conditions. This has been developed as a test for heparin-induced activity, and is being reported in detail elsewhere(4).

---

* This work was supported in part by the U. S. Atomic Energy Commission and by the Lederle Laboratories Division, American Cyanamid Co.

As an initial step in investigating the nature of these reactions, we have carried out chemical studies of the lipid changes brought about in certain lipoproteins by post-heparin plasma. The essential results of these studies are the subject of this paper.

Experimental procedure. Blood was withdrawn from normal human subjects before and after injection of 100 mg of sodium heparin (Lederle). Pre-heparin blood was allowed to clot and the serum centrifuged at low speed to remove any residual red blood cells. Post-heparin blood was centrifuged at 4000 rpm to obtain clear plasma. Egg lipoprotein (ELP) was prepared by a centrifugal isolation method described elsewhere(4). The concentration of this preparation (ELP syrup, usually about 25% lipoprotein) was determined in the analytical centrifuge, and an appropriate dilution made with phosphate buffer (pH = 8.0, ionic strength = 0.1). The final lipoprotein concentration is about 5 to 10%. In general, 1 ml of diluted ELP was incubated with 2 or 3 ml of serum or plasma at 37°C for periods ranging from ½ hour to 8 hours. The incubations were performed with parallel sets of samples, one set containing pre-heparin serum, the other post-heparin plasma from the same individual. At the end of the desired time interval, the reaction was stopped by the addition of 3 ml of methanol and 3 ml of water with stirring. The contents of the reaction tube were quantitatively transferred to a glass-stoppered 100 ml glass cylinder for extraction of lipids. The mixture was extracted twice with 20 ml portions of ethyl ether to remove most of the lipids. The residual aqueous phase was next acidified to pH 2 by gradual addition of 6 N HCl, 3 ml of methanol were added, and 2 more extractions were made with ethyl ether. All of the ether extracts were combined and the solvents were removed by evaporation. The guiding principles in this procedure, wherein most of the lipids are extracted prior to acidification, were to achieve quantitative removal with minimum hydrolysis of lipids to yield fatty acids. While we have demonstrated that hydrolysis of glycerides and cholesteryl esters is negligible under the usual conditions of extraction from acid or alkaline media, the situation with regard to phospholipids remains uncertain. Our precautions are concerned mainly with guarding against possible hydrolysis of this class of compounds, and are prompted in part by inconsistent results obtained in earlier experiments. In order to titrate free fatty acids, the dried extract was dissolved in about 60 ml of low-boiling petroleum ether and the solution filtered into a 100 ml glass-stoppered cylinder. Removal of any trace of the hydrochloric acid which may have remained was accomplished by washing the solution 4 times with distilled water. (This was found to be sufficient to reach the same pH value as a similar system to which no hydrochloric acid had been added. Petroleum ether is preferred for this purpose since hydrochloric acid is removed from it more easily than from ethyl ether.) The solvent was evaporated, the residue was dissolved in about 40 ml of ethyl alcohol, and the solution boiled for one minute to expel carbon dioxide. The hot solution was titrated with sodium hydroxide (0.01 N in 90% ethanol aqueous) to a phenolphthalein end point. This procedure gave satisfactory agreement with titration at room temperature under a nitrogen atmosphere, and the end point determination was checked with a Beckman Model H pH meter (glass electrode). Simplicity of operation dictated the choice of the procedure described. The fatty acids were calculated from the titration as oleic acid (molecular weight = 282). The selection of oleic acid as a reference standard is arbitrary, and is based on the assumption that the fatty acids being measured are predominantly long chain. This follows from the observation that they are semi-solid when isolated, and have the infrared spectral characteristics of long-chain fatty acids. The presence of a minor fraction of low molecular weight acids would not invalidate our basic conclusions. The estimated error in titration is about ±0.5 mg.

Infrared analytical methods. The methods of lipid analysis by infrared spectrophotometry will be reported elsewhere in greater detail, but the essential outline of the procedures as applied in this investigation will be given here. The spectral measurements were made with a Baird Associate Model B double beam recording spectrophotometer equipped with a sodium chloride prism. The absorption cell had a capacity of 0.15 ml and an optical path of 0.9 mm. The identity of the titrable material as fatty acid was confirmed by its infrared spectrum, and in many instances quantitative infrared measurements were carried out in parallel with titrations. In general, the absolute values for fatty acid were slightly lower than those obtained by titration, but the *differences* resulting from pre- and post-heparin incubations were substantially the same by both methods. The infrared measurements were carried out by dissolving the isolated fatty acids in small measured volumes of carbon disulfide and recording the spectrum of the solution. The peak absorbance (logarithm of the reciprocal transmittance) of the carboxyl absorption band at 5.9 microns was measured and converted to fatty acid concentration by means of a calibration curve (absorbance vs. concentration) which had been determined previously with pure oleic acid. In cases where neutral material (esters) remained as a contaminant of the separated fatty acids, its presence could be detected in the spectrum and an appropriate correction applied. In analyzing the total lipid extract from an incubation mixture to determine fat and in some instances other lipids as well, a separation was carried out by means of a silicic acid adsorption column in the manner described by Borgstrom(5). The lipid mixture was dissolved in petroleum ether, put on the adsorption column, and eluted in 3 fractions by the following sequence of solvents: I—5% chloroform in petroleum ether (combined with petroleum ether from original solution); II—chloroform; III—methanol. The principal lipids are distributed in these 3 fractions as follows: I—cholesteryl esters; II—fat (triglycerides), free fatty acids, unesterified cholesterol; III—phospholipids. The eluted solutions were evaporated to dryness, the residues were redissolved in measured volumes of carbon disulfide, and spectra were recorded of the resulting solutions. In each of these 3 spectra, the ester carbonyl absorption band at 5.8 microns can be used to measure a different lipid component. (In Fraction III, it represents the sum of lecithins and cephalins, but sphingomyelins do not contribute to this band(6).) If free fatty acids are present in Fraction II in detectable amounts, a correction can be made. Unesterified cholesterol requires no correction for amounts which can conceivably be present. It has been shown by experiment that partial glycerides, which may be presumed to be present as hydrolysis products in certain cases, do not accompany triglycerides in the chloroform eluate, but remain on the column and are

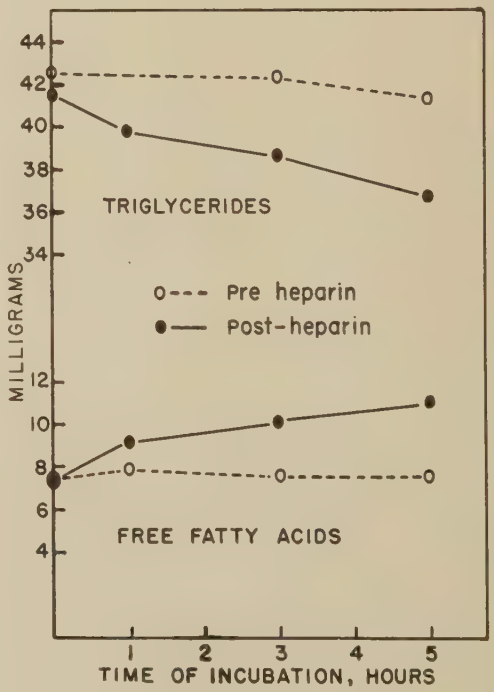


FIG. 1. Lipid changes on incubation of egg lipoprotein (1 ml) with 2 ml of pre-heparin serum or post-heparin plasma from Subject No. 1. (Triglycerides as olive oil; fatty acids as oleic.)

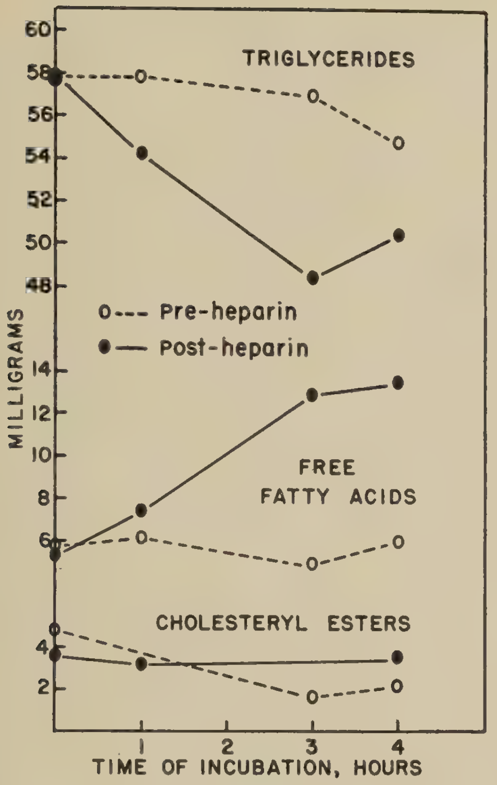


FIG. 2. Lipid changes on incubation of egg lipoprotein (1 ml) with 3 ml of pre-heparin serum or post-heparin plasma from Subject No. 2. (Triglycerides as olive oil; fatty acids as oleic; cholesteryl esters as stearate.)

partially eluted by methanol. The reference materials used for intensity calibrations were: I—cholesteryl stearate, synthesized by direct esterification(7); II—edible olive oil (commercial); III—egg lecithin, reprecipitated several times with acetone. Statistical evaluation of these methods is not complete, but on the basis of column recoveries, instrumental error, etc., we believe that they are accurate to within about ± 10%. In a series of duplicate analyses of various lipoproteins, the triglyceride values disagreed by 0.0, 1.1, 10.0, 2.5, and 1.2%. To a considerable extent we have relied on the reproducibility in several experiments and comparison with controls as criteria of the validity of the observed differences.

Results and discussion. Some typical graphs, Fig. 1, 2 and 3, illustrate the essential findings with respect to fatty acid release and other lipid changes in the type of incubation experiment described. The liberation of fatty acids from lipoprotein substrates by the action of post-heparin plasma, as contrasted with a minimal effect with pre-heparin plasma, has been observed in many other such experiments. Lipemic serum or an appropriate ultracentrifugal fraction thereof containing low density lipoproteins can also serve as a substrate for the reaction. The source of the fatty acids is principally the triglycerides as evidenced by a decrease in their amount which corresponds roughly to the amount of fatty acids released. The phospholipid (sphingo-

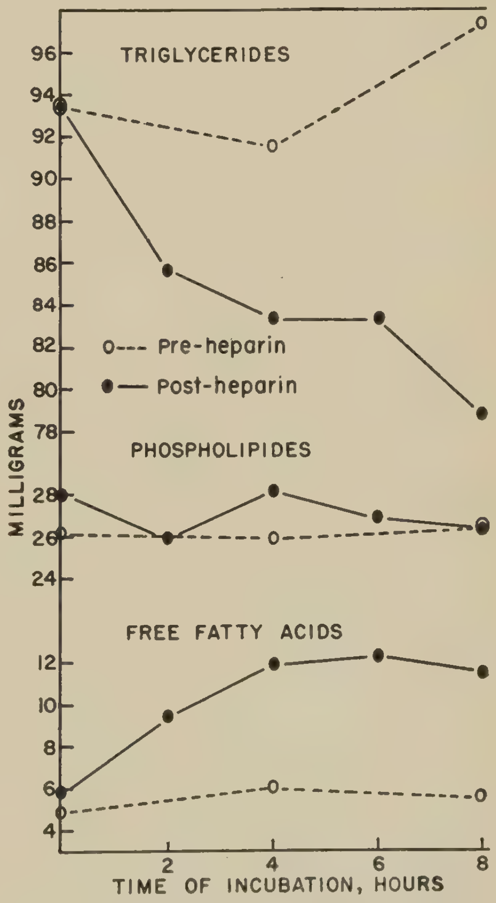


FIG. 3. Lipid changes on incubation of egg lipoprotein (1 ml) with 3 ml of pre-heparin serum or post-heparin plasma from Subject No. 3. (Triglycerides as olive oil; phospholipids as lecithin—sphingomyelin not included in measurement; fatty acids as oleic.) In this particular experiment the cholesteryl esters were not separated from the triglyceride fraction and presumably contribute a small constant error in these curves.

myelin not included) curves (Fig. 3) show no divergence greater than the experimental error. Analyses of the cholesteryl esters are somewhat erratic but indicate no hydrolysis. Complete hydrolysis of the total amount present would account for only about 25% of the free fatty acid observed.

The release of fatty acids could not be effected with pre-heparin serum by adding heparin prior to incubation. Nor was any reaction observed with post-heparin plasma which had first been heated to 58°C for 10 minutes. In order to explore the enzymatic nature of the process further, some experiments were performed with various inhibitors of the type commonly employed in metabolic studies. The following substances at concentrations of 0.02 M in the incubation mixture showed less than 10% inhibition (as determined by titration): sodium cyanide, sodium fluoride, sodium azide, sodium iodoacetate, and sodium oxalate. Data with 2,4-dinitrophenol were uncertain because of interference with the measurements. Sodium arsenite at the same concentration caused 60-70% inhibition throughout a period of 1-24 hours.

Further studies of fatty acid release in this type of system are now in progress, with these and other inhibitors as well as activators, in an effort to develop the kinetic and enzymatic relationships.

The process of lipoprotein alteration that is initiated in the body by heparin is certainly more complex than the straightforward lipolysis we have observed *in vitro*. Nevertheless, a selective breakdown of triglycerides is consistent with the disappearance of part or all of the faster-floating species of lipoproteins, in which fat is a predominant component. If hydrolysis of this fat is accompanied by removal of the products from the lipoprotein, the residual portion of the molecule may simply appear as a higher density species in the ultracentrifuge pattern. Thus the lipolysis reaction could conceivably be a direct cause of the observed lipoprotein transformations; but the experimental results thus far are not sufficient to demonstrate such a relationship.

The clearing of turbidity in solutions of egg lipoprotein on incubation with post-heparin plasma could also be explained by lipolysis. This reaction yields products—fatty acids and partial glycerides—which are more hydrophilic than the original material. And the resulting change in the colloidal character of the system would tend toward a greater degree of solubilization.

Summary. 1. Plasma from human subjects who have received intravenous heparin a short time previously, when incubated with certain lipoproteins for period of 4 to 8 hours at 37°C, has been found to cause partial hydrolysis of the glyceride component of the lipoprotein with concomitant release of fatty acids. Sera from the same individuals before heparin administration did not cause measurably real hydrolysis under the same conditions. 2. Heating the plasma before incubation prevents the reaction, but of a number of common enzyme inhibitors tried, only sodium arsenite is effective. 3. Our results are consistent with—and may at least partially explain—certain other lipoprotein changes which may be classed as heparin effects, *e.g.*, the observed redistribution of lipoproteins shown by the ultracentrifuge, and the cleaning of turbidity in egg lipoprotein solutions.

We wish to thank Dr. John W. Gofman and Dr. Hardin B. Jones for their advice and interest in this work.

---

1. Hahn, P. F., *Science,* 1943, v98, 19.

2. Graham, D. M., Lyon, T. P., Gofman, J. W., Jones, H. B., Yankley, A., Simonton, J., and White, S., *Circulation,* 1951, v4, 666.

3. Anfinsen, C. B., Boyle, E., and Brown, R. K., *Science,* 1952, v115, 583.

4. Nichols, A. V. and Rubin, L., To be published.

5. Borgstrom, B., *Acta Physiol. Scand.,* 1952, v25, 101, 111.

6. Freeman, N. K., Lindgren, F. T., Ng, Y. C., and Nichols, A. V., *J. Biol. Chem.,* In press.

7. Page, I. M., and Rudy, N., *Biochem. Z.,* 1930, v220, 304.

Received May 4, 1953. P.S.E.B.M., 1953, v83

Retention of Co 60 Labeled Vitamin B_{12} in Chickens.* (20313)

J. T. Jackson, L. J. Machlin, E. A. Brandenburger, W. L. Kellogg, and C. A. Denton. (Introduced by N. R. Ellis.)

From the Bureau of Animal Industry, U. S. Department of Agriculture, Beltsville, Md.

It has been reported from this laboratory (1) that injection of crystalline vit. B_{12} into eggs, deficient in this vitamin, results in a growth response in the chicks hatched from these eggs which is sustained for at least 12 weeks. This raises the question as to whether this prolonged growth response is due to an initial growth impetus or to the retention of the vitamin throughout the experimental period.

The present work was planned to determine whether or not a significant quantity of vit. B_{12} was retained in the chick over a 12-week period when the Co 60 labeled vitamin was administered by injection into vit. B_{12}-deficient eggs.

Experimental. Eggs used for injection were from Rhode Island Red hens fed a vit. B_{12}-deficient diet. The eggs were injected on the 6th day of incubation as follows: One lot was injected with 3 μg of Co 60 labeled vit. B_{12} which had a specific activity of about 38 μc per mg; a second lot was injected with 3 μg of the unlabeled vitamin (Cobione); a third lot was not injected. Chickens hatched from all the eggs received a corn-soybean meal diet which was low in vit. B_{12}. At least 5 birds from each group were sacrificed at one day, 3, 6, 9, and 12 weeks of age. The chickens from the B_{12}-injected groups demonstrated a growth response similar to that obtained by Lillie *et al.*(1). *Radioactivity measurements.* Individual, whole chickens were wet ashed, made to volume and their radioactivity determined with a proportional counter. All samples were counted for a sufficient length of time to insure a counting error of not more than 5%. All results were referred to a standard prepared in the same manner as the samples. Self absorption and decay corrections were applied in all cases. The samples were prepared for counting by direct evaporation of the nitric acid digests in stainless steel cups. Twenty chicks from the radioactive group were kept in a separate battery and the droppings collected quantitatively for each of the 12 weeks of the experimental period. Each weekly collection of droppings was dried, ground and a representative sample wet ashed and made to volume. *Microbiological assay.* The B_{12} content of chickens from the eggs injected with Cobione was estimated by the U. S. Pharmacopeia method(2), using cyanide in the extraction procedure(3). In order to measure only the B_{12} content in the tissues of the chicken, the intestinal contents were discarded. The chickens were skinned in order to facilitate easy removal of the feathers.

Results and discussion. The results of radioactivity measurements and microbiological assays on whole chicks for the various age groups are reported in Table I. The vit. B_{12} values of the measured radioactivity are reported, assuming that there was no degradation of the vitamin after injection. In support of this assumption Rosenblum and coworkers (4) have concluded, on the basis of parallel radioactivity and microbiological assays, that the injected radiovitamin is excreted as such in the urine of the rat.

The results indicate that there was a rapid initial loss of radioactivity during the first 3 weeks, after which there was a rather uniform and continuous loss for the remainder of the experimental period. Monroe and coworkers(5) have reported, on the basis of a short term experiment, that B_{12} has a low turnover rate in week-old chicks when the vitamin is injected intraperitoneally. The results reported here confirm this low turnover rate for B_{12}. In addition, the data show that as much as 30% of the injected vitamin is retained by the chick over a 12-week period.

The vit. B_{12} values for the droppings and

---

* This project was supported in part by the U. S. Atomic Energy Commission. A preliminary report was presented at annual meeting of American Institute of Nutrition, April 7, 1953.

TABLE I. Vitamin B_{12} Values for Chicks and Droppings Estimated by Microbiological Assay and Calculated from Radioactivity Measurements.

Age	B_{12} values estimated by microbiological assay, μg	B_{12} values calculated from radioactivity		
		Per chick, μg	Cumulative excretion per chick, μg	% recovery
(Egg)	3.12*	3.0	—	100.0
1 day	3.03*	2.46	—	—
3 wk	1.20	1.71	.94	88.5
6 ”	1.61	1.42	1.65	102.2
9 ”	2.18	1.23	—	—
12 ”	4.10	.98	—	—

* 3 μg inj. B_{12} + B_{12} normally present.

the percent recovery as determined by adding the total B_{12} excreted to that retained by the chicks are shown in the last 2 columns in Table I. Essentially complete recovery of the radioactivity was obtained for the 3- and 6-week intervals. Although droppings were collected weekly for the entire experimental period, values are reported only for the first 6 weeks because of the large dilution of the small amount of radioactivity in the later samples.

A comparison of the values reported, based on the 2-assay methods, suggest that there was no appreciable uptake of the vitamin from the basal diet or intestinal synthesis during the first 6 weeks of the experiment, since the data are in approximate agreement for these age groups. Beyond 6 weeks of age there is a decided increase in the B_{12} values based on microbiological assay compared to those calculated from radioactivity, indicating absorption of the vitamin from some exogenous source. The difference in the chick B_{12} values noted at 3 weeks is of doubtful significance and probably represents experimental error in one or both of the assay methods. However, the possibility that this difference represents Co 60 in some form other than vit. B_{12} can not be ruled out.

Summary. The results indicate that over 30% of the injected Co 60 activity is retained for as long as 12 weeks in chicks hatched from eggs injected with the radiovitamin. The total B_{12} content of the chick as measured microbiologically roughly parallels Co 60 activity until 6 weeks of age, after which there is a decided increase in the total B_{12} content of the chicks.

---

1. Lillie, R. J., Olsen, M. W., and Bird, H. R., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 598.

2. Vitamin B_{12} Activity Assay, Third Supplement to the Pharmacopeia of the United States, Fourteenth Revision (1950).

3. Denton, C. A., Kellogg, W. L., and Bird, H. R., *Federation Proc.*, 1952, v11, 440.

4. Rosenblum, C., Chow, B. F., Condon, G. P., and Yamamoto, R. S., *J. Biol. Chem.*, 1952, v198, 915.

5. Monroe, R. A., Patrick, H., Comar, C. L., and Goff, O. E., *Poultry Sci.*, 1952, v31, 79.

Received May 8, 1953. P.S.E.B.M., 1953, v83.

Bioassay of Parathyroid Hormone.* (20314)

BETTY L. RUBIN† AND RALPH I. DORFMAN.†

From the Departments of Biochemistry and Medicine, Western Reserve University School of Medicine and Lakeside Hospital, Cleveland, O. and the Worcester Foundation for Experimental Biology, Shrewsbury, Mass.

Tweedy, Chilcote, and Patras(1) demonstrated an increase in radiophosphorus excretion in thyroparathyroidectomized rats following the injection of 5 or more U.S.P. units of parathyroid hormone and 3 mg of phosphorus as disodium phosphate (13.75 mg of disodium phosphate). These studies are concerned with the use of this increase in P^{32} excretion as the basis of an assay technic for parathyroid hormone.

Animals, materials and methods. Parathyroid extract (Lilly and Co.) was administered to 90-160 g, thyroparathyroidectomized Holtzmann albino rats maintained on a dog chow diet. The hormone was administered subcutaneously in 1 cc of saline. Optimum conditions with respect to the amount of disodium phosphate given and route of administration were studied, but in all experiments the material was injected in a volume of 2 cc. After both hormone and phosphate had been administered, the animals were immediately placed in glass metabolism cages and the urine collected quantitatively for 3 hours. The method of collection was the same as that used by Dorfman, Potts, and Feil(2). *Experimental.* In order to establish the optimal conditions for the assay, the following variables were investigated: 1) The interval between thyroparathyroidectomy and use of animals for assay of parathyroid activity, 2) the amount of disodium phosphate to administer, 3) the route of administration of disodium phosphate, and 4) the effect of varying the time interval between the hormone and phosphorus and the collection of urine.

Influence of interval between operation and assay. The effects of differing intervals between operation and assay are shown in Table I. In the first 2 experiments, 13.75 mg of disodium phosphate per 100 g of body weight were administered to animals 14 and 32 days after operation, and 1.55 mg of disodium phosphate per 100 g of body weight were administered to the same animals a week later. Five U.S.P. units caused a significant increase in P^{32} excretion in 2 of the 4 trials. In the third and fourth experiments, 1.2 mg of disodium phosphate were administered to rats one day after operation and again a week later. One-half to 10 U.S.P. units caused a significant increase in P^{32} excretion in the animals one day after operation. Fisher's t values(3) were used as criteria of the significance of the differences between the mean excretions of control and experimental groups. Using a total of 10 animals, the t value must be greater than 2.28 for the probability (P) to be less than 0.05. With a total of 20 animals, t must be greater than 2.09, and for 26 animals, t must be greater than 2.05. The data also show that 5 U.S.P. units administered to animals 14-39 days after operation caused an increase in P^{32} excretion of lesser magnitude than the increase caused by 2.5 units administered one day after operation. A tendency for the excretion of P^{32} in the control group to increase with increasing time after operation was observed. This is especially striking an in experiment (the sixth in Table I) in which animals selected for their low excretion of phosphorus on the first post-operative day (mean = 1.21 ± 0.23%) were tested again on the second day, at which time the mean P^{32} excretion had increased to 5.66 ± 1.21%. No significant difference in P^{32} excretion of the untreated operated animals could be demonstrated within the first 16 hours post-operatively (Table I). The individual variations were high, as indicated by the large standard errors of the means. In all later experiments animals were used within 24 hours after operation.

---

* Supported in part by a grant from the Division of Grants and Fellowships, National Institutes of Health, U. S. Public Health Service.

† Present address: Worcester Foundation for Experimental Biology, Shrewsbury, Mass.

TABLE I. Effect of Varying the Time Interval between Operation and Start of Test. (P^{32} administered intraperitoneally, simultaneously with hormone.)

Time post-op.	Na_2HPO_4 admin., mg/100 g	Parathyroid hormone, U.S.P. units	Urinary excretion of inj. P^{32}				% change	t
			No. animals	Control, % ± S.E.	No. animals	Exp., % ± S.E.		
* 32 days	13.75	5	5	$6.57 \pm .84$	6	9.36 ± 1.02	+45	2.11
39 ”	1.55	5	5	3.25 ± 1.34	5	11.15 ± 1.57	+243	4.40
* 14 ”	13.75	5	6	6.87 ± 1.31	6	$9.42 \pm .69$	+37	1.71
21 ”	1.55	5	6	5.06 ± 1.65	6	$9.63 \pm .76$	+90	2.54
*1–2 ”	1.15	2.5	11	2.51 ± 1.03	9	10.59 ± 1.80	+321	3.84
		5.0			11	11.62 ± 1.32	+363	5.35
		10.0			9	14.22 ± 1.82	+466	5.57
8–9 ”	1.2	2.5	11	$10.66 \pm .89$	11	12.38 ± 1.45	+16	1.01
		5.0			10	10.26 ± 1.57		.22
		10.0			10	11.73 ± 1.92	+10	.51
* 1 ”	1.2	.5	10	$2.60 \pm .87$	11	5.91 ± 1.10	+127	2.36
		1.0			11	6.09 ± 1.08	+134	2.49
		2.5			9	10.27 ± 2.55	+295	3.19
8 ”	1.2	.5	7	$3.47 \pm .83$	12	5.73 ± 1.35	+65	1.41
		1.0			9	$4.99 \pm .98$	+44	1.17
		2.5			9	6.35 ± 1.33	+83	1.80
* 1 ”	Tracer dose	0	14	$1.95 \pm .67$				
8 ”		0	14	$2.80 \pm .40$				
1 ”	.2	0	6	$1.21 \pm .23$				
† 2 ”		0	6	5.65 ± 1.21				
* 1 ”	1.2	0	8	$3.49 \pm .99$				
2 ”		0	8	$3.96 \pm .21$				
1¼ hr	1.2	0	9	3.82 ± 1.6				
2 ”		0	8	2.52 ± 1.38				
4 ”		0	8	$1.77 \pm .83$				
8 ”		0	8	$1.46 \pm .65$				
16 ”		0	8	3.65 ± 1.74				

* Same animals used at both time intervals.

† Selected animals showing lowest excretion on day 1.

Influence of varying amounts of disodium phosphate. When 13.75 mg of disodium phosphate per 100 g of rat were administered, there was no significant difference in P^{32} excretion between operated and unoperated control animals, both excreting more than 6% of the injected P^{32}. Five units caused the excretion in operated animals to increase approximately 40% over the control excretion.

Influence of varying dose of hormone. Table II lists the responses to various doses of parathyroid hormone when 1.2 mg disodium phosphate per 100 g of body weight were administered simultaneously with the hormone to animals operated 24 hours previously. Table III lists the responses when tracer P^{32} and hormone were administered to animals operated 24 hours previously. A straight line log-dose response relationship fits the data obtained with 1.2 mg disodium phosphate much more closely than that obtained with tracer P^{32}. The equation of the regression line for the data obtained with 1.2 mg phosphate over the dosage range from 0.5 to 3.0 U.S.P. units, determined by the method of Fisher (1934), is $y = 8.3 + 8.6 \log x$. The sensitivity, as indicated by a significant increase in P^{32} excretion is about the same for both levels of phosphate administration, 0.5 U.S.P. unit causing a significant increase (64-220% in magnitude) in P^{32} excretion over that of the controls in 4 of 6 experiments. The variation of P^{32} excretion within groups appears to be somewhat less when 1.2 mg of phosphorus was administered than when tracer P^{32} was used.

Influence of time interval between injection of hormone plus injection of phosphorus and

TABLE II. Influence of Parathyroid Hormone on Phosphate Excretion of Thyroparathyroidectomized Male Rats. 24 hr after operation, 1.2 mg Na_2HPO_4/100 g. P^{32} and hormone inj. simultaneously I.P.

Parathyroid hormone, U.S.P. units	No. experiments	No. rats	Mean P^{32} excretion, % ± S.E.
0	11	97	2.68 ± .32
.10	1	10	2.90 ± .50
.25	1	10	2.80 ± .47
.50	3	28	5.35 ± .33
1.00	9	101	8.35 ± .53
2.50	2	18	10.43 ± 1.49
3.00	8	90	12.60 ± .80
5.00	1	11	11.62 ± 1.36
10.0	1	9	14.22 ± 1.82

TABLE III. Influence of Parathyroid Hormone on Phosphate Excretion of Thyroparathyroidectomized Male Rats. 24 hr after operation, tracer P^{32}. P^{32} and hormone inj. simultaneously I.P.

Parathyroid hormone, U.S.P. units	No. experiments	No. rats	Mean P^{32} excretion, % ± S.E.
0	4	34	2.40 ± .39
.5	2	23	6.58 ± .90
1.0	1	16	3.62 ± .80
1.5	1	16	7.91 ± 1.71
2.0	1	16	10.78 ± 1.36
3.0	1	13	6.17 ± .63
6.0	1	16	9.74 ± 1.58

TABLE IV. Influence of Parathyroid Hormone on Phosphate Excretion of Thyroparathyroidectomized Male Rats. 2-4 hr after operation, 1.2 mg Na_2HPO_4/100 g. 3½ hr between P^{32} (SQ) and hormone.

Parathyroid hormone, U.S.P. units	No. experiments	No. rats	Mean P^{32} excretion, % ± S.E.
0	7	68	.15 ± .05
.25	1	10	.49 ± .21
.50	1	7	.62 ± .22
1.00	3	30	.59 ± .14
4.00	1	10	1.92 ± .39
16.00	1	10	2.58 ± .24

collection of urine. In the experiments described so far, the phosphate was injected into the animals immediately after the administration of the hormone, and the urine was collected in the 3 hours immediately following. The data from another experimental design are presented in Table IV. Animals were used 2 to 4 hours after operation. After the administration of phosphate, the urine was collected for 3½ hours and discarded. Then the hormone or saline was administered and the urine collected for 3 hours. In Table IV are listed the excretions of P^{32} at various levels of parathyroid hormone treatment. 0.5 U.S.P. unit was the minimum effective dose. A straight line relationship was found to hold over the range of 0.25 to 16 U.S.P. units. The P^{32} excretion values are lower and the slope is less steep than that observed when the same amount of phosphate (1.2 mg) was administered, simultaneously with the hormone, to animals operated 24 hours previously.

Discussion. Excretion of P^{32} by thyroparathyroidectomized rats furnishes a sensitive indicator for parathyroid activity. In spite of the high variation in the P^{32} excretion of untreated animals, 0.5 U.S.P. unit caused a significant increase in P^{32} excretion as compared to solvent-treated controls. The most sensitive method in the literature (Tepperman *et al.*(4)) requires 12.5 U.S.P. units for a minimum significant effect on the blood phosphate level in the rat. Another advantage of the method described is its comparative ease.

The slope is steeper for the log dose-response curve obtained with the technic in which animals were used 24 hours after operation and hormone and 1.2 mg of disodium phosphate were administered simultaneously than for the technic in which 3½ hours elapsed between phosphate and hormone injection.

Summary. There is an increase in excretion of administered P^{32} by thyroparathyroidectomized male rats under the influence of parathyroid hormone. Amounts as small as 0.5 U.S.P. unit may be detected, an increase in sensitivity of 25 times over the method of Tepperman *et al.*(4), and of 200 times over the standard U.S.P. assay method. In addition, there are no tedious chemical determinations of blood phosphate levels to be done.

---

1. Tweedy, W. R., Chilcote, M. E., and Patras, M. C., *J. Biol. Chem.*, 1947, v168, 597.

2. Dorfman, R. I., Potts, A. M., and Feil, M. L., *Endocrinology*, 1947, v41, 464.

3. Fisher, R. A., *Statistical Methods for Research Workers*, London, Oliver and Boyd, 1934.

4. Tepperman, H. M., L'Heureux, M. V., and Wilhelmi, A. E., *J. Biol. Chem.*, 1947, v168, 151.

Received May 8, 1953. P.S.E.B.M., 1953, v83.

Excretion of Cobalt^{60}-Labeled Vitamin B_{12} after Total Gastrectomy.* (20315)

MARIAN E. SWENDSEID, JAMES A. HALSTED, AND RAYMOND L. LIBBY.

From the Veterans Administration Center, and the Departments of Medicine and Radiology, University of California Medical Center, Los Angeles.

The development of a macrocytic anemia in persons without gastric tissue has been attributed to removal of a source of Castle's intrinsic factor(1,2). There is now evidence, from studies in patients with pernicious anemia, that the role of intrinsic factor is to potentiate the hemopoietic activity of orally administered vit. B_{12}(3,4). More specifically, Heinle and coworkers found that Co^{60}-labeled vit. B_{12} was not absorbed when administered orally to patients with pernicious anemia unless a source of intrinsic factor was given simultaneously(5). It therefore seemed pertinent to study the absorption of vit. B_{12} in a series of totally gastrectomized subjects and to observe the possible effect of simultaneous administration of intrinsic factor as present in gastric juice.

Clinical material and methods. Four patients who had been subjected to a total gastrectomy for carcinoma of the stomach were studied. In each instance from 0.5 to 1 cm of the distal esophagus and from 2.5 to 5 cm of proximal duodenum were removed along with the entire stomach. The spleen was removed in all cases, and from one-third to one-half of the pancreas (tail and body) in cases 1, 2, and 4. A jejunal transplant to act as a "substitute stomach" or holding reservoir was inserted between esophagus and duodenum in these 3 cases. No metastases were found at operation except one positive regional node in Case 1, and there was no clinical evidence of metastatic carcinoma in any of the cases at the time of this study. Other relevant data in each case include the following: Case 1. M.E., a male, aged 57, had a total gastrectomy 3 months ago. There was no anemia either before operation or at the time of study. He has had no injections of vit. B_{12} or liver extract. Sternal biopsy showed a normoblastic marrow. Weight before operation was 121 lb and at present is 106 lb. He feels entirely well and eats a full diet without digestive complaints, but has large, greasy stools 2-3 times daily. Case 2. F.P., a male, aged 60, had a total gastrectomy 10 months ago. He had no anemia before operation but a mild anemia was detected 3 months before this study. (R.B.C. was 3.4 mil. per cmm; Hgb, 12.6 g per 100 ml). The bone marrow was normoblastic. Vit. B_{12} injections resulted in improved appetite and strength and a 5 lb gain in weight. He weighed 135 lb before operation and weighs 120 lb at present. He has an excellent appetite without digestive complaints, but the stools (1-2 daily) are large and greasy. Case 3. G.S., a male, aged 43, had a total gastrectomy 6 years ago. There has been no anemia at any time and sternal marrow is normal. He received liver extract for 2 months after operation, but none since and no vit. B_{12}. His weight before operation averaged 150 lb, but since then he has never weighed more than 120 lb. He has excellent appetite without digestive difficulties except for diarrhea for a day or 2 about once a week. Case 4. W.V., male aged 32, had a total gastrectomy 9 months ago. There has been no anemia at any time, and he has had no injections of vit. B_{12} or liver extract. He feels entirely well and is without digestive complaints. He has 1-2 formed stools daily, which are fatty, and he has maintained his normal weight of 145 lb. 0.5 γ doses of the Co^{60}-labeled vit. B_{12}† containing 0.046 μc of radioactivity, were administered orally to the subjects in 2 or more successive tests, either alone or with added neutralized gastric juice from normal or duodenal ulcer patients. All stools were collected daily for a period of 4

---

* This study was supported in part by a grant from Parke, Davis and Co., Detroit, Mich.

† We are indebted to Dr. Charles Rosenblum of Merck and Co. for generous supplies of this material.

TABLE I. Fecal Excretion of Co^{60} Following Oral Administration of Radioactive Vit. B_{12} to Persons with a Total Gastrectomy; 4 Cases.

Dose	% Co^{60} excreted Test 1	% Co^{60} excreted Test 2
.5 μg B_{12}	103	96
.5 μg B_{12} + 50 ml gastric juice	12	17
.5 μg B_{12}	96	94
.5 μg B_{12} + 100 ml gastric juice	6	—
.5 μg B_{12}	86	89
.5 μg B_{12} + 50 ml gastric juice	64*	18
.5 μg B_{12}	98	—

* Patient became ill with diarrhea on day following test dose.

days after administration of the test substances. Each day's stools were kept separate, dried on the steam bath and ashed in a muffle furnace. The ash was transferred to an ointment tin, made up to a constant weight of 25 g, and measured for gamma radioactivity in a scintillation counter(6). The amounts found in each day's stool were cumulated to determine the total excretion of Co^{60}.

Results. The test method developed by Heinle and his associates(5) for measurement of vit. B_{12} absorption was found to be eminently satisfactory as used under the conditions described above. Confirmation of their results in pernicious anemia has been obtained by us. In 3 such patients, all of whom were in hematologic remission, from 89 to 100% of the administered radioactivity was recovered in the stool samples. Control individuals excreted approximately 25% with variations in some subjects depending on whether the vitamin was given at meal time or between meals.

Data on 4 persons with a total gastrectomy receiving radioactive vit. B_{12} are recorded in Table I. In all cases, from 86 to 100% of the administered radioactivity was excreted in the stool samples, indicating a condition of negligible absorption of vit. B_{12}. This non-absorption occurred whether the vitamin was given with meals as in Cases 2 and 3, or between meals in the inter-digestive phase, as in Cases 1 and 4. When gastric juice was administered with vit. B_{12}, the excretion of radioactive Co^{60} dropped to levels similar to those obtained in control individuals, except in one instance where the patient was ill.

Discussion. The above experiments indicate that patients who have had a total gastrectomy do not absorb orally administered vit. B_{12} and therefore cannot depend on dietary sources to supply their metabolic requirement for this vitamin. Each of the patients studied apparently had steatorrhea and in 3 cases there was inability to regain normal weight despite adequate caloric intake. These facts suggest a general defect in intestinal absorption. However, such an explanation for the non-absorption of vit. B_{12} does not appear to be tenable since absorption of the vitamin did occur when gastric juice was given simultaneously. This observation, when considered in conjunction with the experiments of Heinle *et al.*(5) in pernicious anemia patients, indicates that patients with a total gastrectomy do not absorb vit. B_{12} because of a lack of intrinsic factor. It adds evidence to the original observations of Castle(7) that the gastric mucosa is the principal source of intrinsic factor, although other interpretations are possible.

Reports in the literature show that a macrocytic anemia can develop in patients after a total gastrectomy but that this is not the invariable result, even after long periods of time (8-10). Since it has been the consistent finding of the present study that vit. B_{12} is not absorbed in such patients it would appear, as Ungley(2) has suggested, that other factors besides deprivation of dietary vit. B_{12} may operate in the production of the anemia when it does occur.

Summary. Co^{60}-labeled vit. B_{12} was administered orally in 0.5 γ doses to 4 patients who had had a total gastrectomy. Each patient excreted in the stools essentially all of the administered radioactivity. When gastric juice was given with the vitamin there was a decrease in Co^{60} excretion to levels similar to those found in control subjects.

We gratefully acknowledge the assistance of Mrs. Elizabeth Hvolboll, Miss Panchita B. Thomas, and Mr. Frank Cramer.

---

1. Bethell, F. H., Sturgis, C. C., Rundles, R. W., and Meyers, M. C., *Arch. Intern. Med.*, 1945, v76, 239.

2. Ungley, C. C., *Brit. J. Nut.*, 1952, v6, 299.

3. Berk, L., Castle, W. B., Welch, A. D., Heinle,

R. W., Anker, R., and Epstein, M., *New Eng. J. Med.*, 1948, v239, 911.

4. Hall, B. E., Morgan, E. H., and Campbell, D. C., *Proc. Mayo Clin.*, 1949, v24, 99.

5. Heinle, R. W., Welch, A. D., Scharf, V., Meacham, G. C., and Prusoff, W. H., *Trans. Assn. Am. Phys.*, 1952, v65, 214.

6. Libby, R. L., Taylor, C. M., and Thomas, P. B., *J. Lab. and Clin. Med.*, 1952, v40, 641.

7. Castle, W. B., Townsend, W. C., and Heath, C. W., *Am. J. Med. Sci.*, 1930, v180, 305.

8. Conway, N. S., and Conway, H., *Brit. Med. J.*, 1951, Jan. 27, 158.

9. MacDonald, R. M., Ingelfinger, F. N., and Belding, H. W., *New Eng. J. Med.*, 1947, v237, 887.

10. Hall, B. E., *J.A.M.A.*, 1953 v151, 1.

Received May 11, 1953. P.S.E.B.M., 1953, v83.

Urinary and Fecal Coproporphyrin Excretion in Rats: I. Results in Normals and Castrates.* (20316)

F. W. HOFFBAUER, C. J. WATSON, AND SAMUEL SCHWARTZ.

From the Department of Medicine, University of Minnesota School of Medicine, Minneapolis.

An increase in the excretion of urinary coproporphyrin has been referred to frequently as a sensitive index of liver functional impairment(1). In view of the extensive employment of the white rat in laboratory studies of liver damage, an investigation of this animal's excretion of coproporphyrin was conducted. As a preliminary step, it was necessary to determine the pattern of excretion in the normal well-fed animal. In the course of these investigations, normal male animals were observed to excrete more coproporphyrin in the urine than did females. Accordingly, the effects of castration and the influence of androgenic and estrogenic substances were studied. Since coproporphyrin is excreted both in the urine (UCP) and the feces (FCP), simultaneous measurements of each were made in all experiments.

Methods. In all the experiments, white rats of a uniform strain (Sprague-Dawley) were employed. The animals were fed a stock diet (Purina Fox-chow) *ad libitum* and had free access to water. The animals under study were housed in individual metal cages. In all experiments, 48 hour collections of urine and feces were made for the coproporphyrin analyses. During the collection periods, the rats were housed in individual cages suspended over large glass funnels (25 cm diameter). The associated all-glass unit used for separation of urine and feces was essentially similar to that described originally by Gross and Connel(2). The successful separation of these two forms of excreta was deemed of utmost importance in a study that involved the separate determination of a pigment common to both. The collections were interrupted for 60 minutes at the end of the first 24 hours to permit feeding of the animal and to store the first day's urine and fecal specimens. The sides of the funnels were rinsed with 10 ml of water at the end of each 24-hour period. The washings, the urine and the feces were immediately refrigerated. No preservatives were employed. The test tubes that received the voided urine were painted black to prevent exposure of the contents to light. The urine and the washings were stored in brown bottles while in the refrigerator. The urine and the washings for the 48-hour period were pooled for analysis. Although average values are given in most of the data reported, individual analyses of the 48-hour urine and fecal collections of each rat were made. Plastic cages with glass rod bottoms were used in the early experiments in order to avoid contact of the urine with metal. This was done to reduce the tendency to form metal coproporphyrin complexes. However,

---

* Supported in part under contracts from the Office of the Surgeon General, U. S. Army, under the sponsorship of the Commission on Liver Disease, Armed Forces Epidemiological Board, and the Atomic Energy Commission; and by a grant from the U.S.P.H.S..

TABLE I. Urinary and Fecal Coproporphyrin Excretion in Normal (Sprague-Dawley) Rats.

Analysis		Stock A		Stock B	
		Males	Females	Males	Females
UCP	No.	33	31	25	16
	Range	14–67	6–30	18–70	14–52
	Mean	40 ± 16	14 ± 5	47 ± 13	30 ± 12
FCP	No.	32	31	13	16
	Range	11–91	12–102	16–52	17–45
	Mean	40 ± 22	46 ± 21	34 ± 11	33 ± 9
UCP/FCP	No.	32	30	12	16
	Range	0.3–2.9	0.1–1.0	0.9–2.2	0.4–1.7
	Mean	1.3 ± 0.7	0.4 ± 0.2	1.7 ± 0.4	1.0 ± 0.4

Urinary (UCP) and fecal (FCP) coproporphyrin values in μg/48 hr.

when metal cages were later employed, it was noted that the only significant metal complex present was that of zinc, and this is completely split by the extraction procedure employed. The copper complex of coproporphyrin comprised less than 5% of the total in those instances in which it was specifically sought. (Its concentration was determined by conversion of the non-fluorescing copper complex to free porphyrin by evaporation of the completely extracted ethyl acetate to dryness and solution of the residue in concentrated sulfuric acid.) The estimation of the fecal coproporphyrin was conducted according to the method of Schwartz and Zagaria(3). This method was slightly modified to permit its use in the analyses of the urine samples. During the analyses of both urine and feces, the initial 1.5 N HCl extracts were each diluted to a concentration of 0.12 N HCl and extracted with chloroform to remove protoporphyrin and deuteroporphyrin. The completeness of the separation of the coproporphyrin from these two porphyrins was checked by examining the red fluorescence of the chloroform washings under filtered near-ultraviolet light[†] (405 mu). The coproporphyrin was quantitated fluorimetrically(4). Coproporphyrin precursor was determined by comparing results obtained with and without an iodine wash(5). Estimation of the coproporphyrin isomer distribution was performed on the pooled final extracts of urine and fecal specimens. Procedure B of the method described by Schwartz and coworkers(6) was employed. Studies to be reported in detail elsewhere have revealed that results obtained with this procedure are dependent to an appreciable extent on the purity of the distilled water used. Appropriate controls were therefore analyzed to establish the slope of the calibration curve obtained under the conditions employed for the unknown samples. Even with these precautions, however, accuracy is reduced in mixtures containing over 75% of type III isomer. Values in the range of 75-100% type III isomer are therefore reported here as having more than 75% type III.

Results. I. Studies of normal rats. A. *Total coproporphyrin excretion:* The data for the urinary and fecal coproporphyrin excretion (UCP and FCP) in μg per 48 hours are summarized in Table I. The earlier studies employed rats obtained from a Minnesota laboratory (Stock A), while more recently the rats have been obtained from a Wisconsin laboratory (Stock B). As seen in Table I, male rats excreted significantly greater amounts of urinary coproporphyrin than did female rats from both stocks. Mean FCP values were approximately equal in the two sexes. The mean UCP/FCP ratios in the different groups ranged from 0.4 to 1.7, being significantly higher in the male rats. The reason for the differences observed between the two stocks (especially in the female UCP) is unknown. No significant correlation was found between weight and UCP excretion, though FCP excretion was reduced in young rats of both sexes. These data are summarized in Table II for rats of Stock A only, since the number and weight distribution of rats of Stock B is insufficient for a similar analysis.

[†] Model BL-2, Blacklight Products, Chicago.

TABLE II. Relationship of Weight to UCP and FCP Excretion in Normal Rats (Stock A).

		UCP		FCP	
Sex	Wt, g	No.	Avg µg/48 hr	No.	Avg µg/48 hr
♂	57–105	6	38	6	29
	195–246	13	38	13	45
	257–370	14	41	13	46
♀	74–115	5	14	7	31
	153–200	16	14	14	49
	219–250	10	13	10	51

B. *Excretion of individual coproporphyrin isomers (I and III):* Previous reports of normal rats urinary coproporphyrin have indicated that it consists predominantly of the type III isomer(7,8). In the present study a pooled 5-day collection of urine from 5 normal adult male rats contained a total of 282 µg of coproporphyrin. Isomer analyses of this porphyrin revealed that more than 75% was type III. This was confirmed by the isolation of the tetramethyl ester, yielding typical rosettes with a melting point of 138-142°C. The pooled feces of the same rats contained 186 µg of coproporphyrin, over 75% of which was shown to be the type III isomer. Again typical rosettes of coproporphyrin III methyl ester were isolated. Similar results were obtained from the pooled feces of 2 adult female rats.

C. *Excretion of urinary coproporphyrin precursor:* In several dozen analyses, no significant amounts of urinary coproporphyrin precursor(5) were found in urine collected in the usual way except in 2 instances, where 6 and 20% of the total porphyrin was in precursor form at the time of analysis. However, by collecting the urine in tubes immersed in dry ice to freeze the urine instantly, coproporphyrin precursor accounted for an average of 33% of the total in 6 female rats and of 24% in 6 male rats. These values are within the range of those found in human urine collected similarly(5).

D. *Excretion of other porphyrins:* Examination of both urine and feces has repeatedly demonstrated the presence of small amounts of an ether-insoluble, uro-type porphyrin. It has not yet been crystallized for more precise identification. No protoporphyrin or other chloroform-soluble porphyrins could be demonstrated in several hundred ml of pooled urine from both male and female rats. Trace amounts may, however, be found in the presence of very slight fecal contamination.

E. *Relationship of urine volume to coproporphyrin excretion:* The relationship of urine volume to the UCP was investigated by inducing a diuresis in rats in which UCP and FCP values had been previously determined. 0.8% saline solution was substituted for drinking water for one week; urine and fecal coproporphyrin analyses were then repeated. The results are presented in Table III. Although the urine volumes increased, the values for the UCP increased but very little. The reason for the drop in FCP during saline diuresis is uncertain.

II. Studies of castrated rats. Since the normal male rat exhibited an average UCP value nearly twice that of the normal female, the effect of castration was investigated. Control values were obtained in 10 rats; the animals were then castrated. In the 39 days that followed, 3 UCP-FCP determinations were made. The average UCP values rose slightly in both groups of animals. Beginning on the 60th day, the 4 male castrated rats re-

TABLE III. Effect of Saline Diuresis on Urinary and Fecal Coproporphyrin Excretion in Normal Rats.

No. rats		Body wt, g	Urine vol, ml/48 hr	UCP, µg/48 hr	Feces wt, g/48 hr	FCP, µg/48 hr	Drinking fluid
6 ♂	Range	250–295	9–46	33–67	3–23	30–69	Water
	Avg	270	20.5	49	7.6	49	
	Range		43–150	34–64	6–18	11–49	.8% saline
	Avg		78	53	8.6	32	
6 ♀	Range	190–207	9–25	6–22	2–11	43–79	Water
	Avg		15.7	11	5.9	64	
	Range		10–78	11–16	4–9.5	29–50	.8% saline
	Avg		42	14	6.0	35	

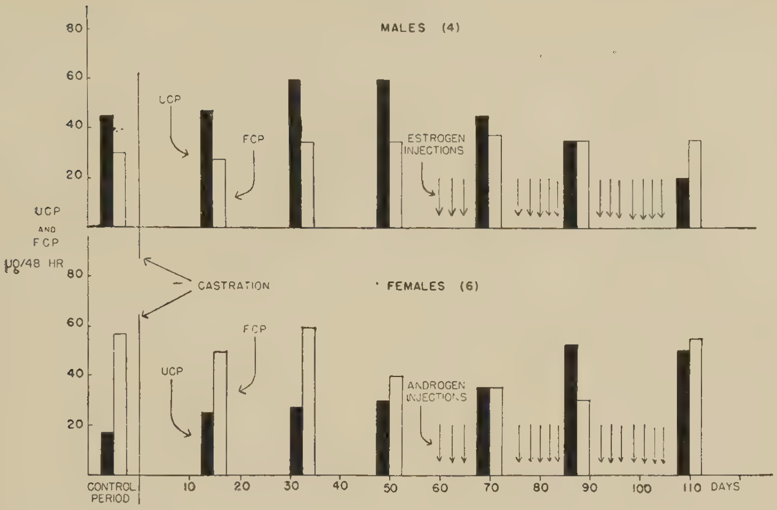


FIG. 1. Effect of castration and administration of estrogen or androgen on excretion of urinary and fecal coproporphyrin in rats.

ceived a series of intramuscular injections of 100 I.U. of an estrogen (Theelin). The 6 female castrates received intramuscular injections of an androgen (0.1 mg testosterone propionate). By the 106th day, 17 injections had been given. At that time the UCP values for the males had decreased from an average control value of 44 μg to 20 μg per 48 hours. The female rats showed an average increase of the UCP from 17 μg to 51 μg per 48 hours. The FCP values in both groups remained fairly constant. The results are shown in Fig. 1.

Discussion. The normal well-fed rat excretes a relatively large amount of coproporphyrin in the urine, average per diem UCP values being comparable to those seen in normal rabbits. The values in normal human adult males, determined by a slightly modified method, average only about 8 times as much, or approximately 160 μg per day(4,9). Though the type I isomer has been shown to be preponderant in human urine(10), the type III isomer predominates in both urine and feces of rats. In this respect, too, the rat is comparable to the rabbit(3).

The variation of the UCP values in the 2 sexes was not anticipated. So far as is known, this difference had not been reported previously, although recent observations in humans, in this laboratory, have indicated significantly lower values in females(9). The significant alteration of the UCP value by castration and estrogen or androgen injections in the present study indicates that observed sex differences are not merely chance observations in the group of animals studied. The mechanism or the site of action of estrogen or androgen in this particular respect is unknown. A primary effect upon the liver would appear to be most likely, since the excretory pattern of coproporphyrin is related to liver functions (11,12) and since the above agents are known to influence some hepatic functions under certain conditions.

Conclusions. 1. Excretion of coproporphyrin in the urine and feces of normal rats has been measured in 2 stocks of Sprague-Dawley rats. Collections periods of 48 hours were made. Male rats of these 2 stocks excreted an average of 40 ± 16 and 47 ± 13 μg of urinary coproporphyrin per 48 hours, respectively, while the female rats excreted an average of only 14 ± 5 and 30 ± 12 μg of urinary coproporphyrin per 48 hours, respectively. There was no significant correlation between body weight and urinary coproporphyrin excretion. No significant sex difference was found in the fecal excretion of coproporphyrin; mean values in the various groups ranged from 33 ± 9 to 46 ± 21 μg per 48 hours. Reduced values were found in both male and female rats weighing less than 115 g. In both males and females, over 75% of the urinary and fecal coproporphyrin was type III isomer. 2. The administration of androgen to female castrate rats increased the excretion of coproporphyrin in the urine. The administration of estrogen to male castrate rats caused a decrease in the amount of coproporphyrin in the urine. 3. A diuresis, induced by substituting saline for drinking water, did not increase the urinary excretion of coproporphyrin. 4. Trace amounts of uro-type porphyrins are excreted normally in rat urine and feces. Protoporphyrin is excreted only in the feces.

The authors gratefully acknowledge the valuable assistance of Miss Bernadine Wittenburg and Mr. Michael Keprios in this study.

---

1. Watson, C. J., and Larson, E. A., *Phys. Rev.*, 1947, v27, 478.

2. Gross, L., and Connel, S. J. B., *J. Physiol.*, 1923, v57, LX.

3. Schwartz, S., and Zagaria, R., *Metallurgical Lab. Rep.* CH-3600 (MDDC-504), Chicago, 1946.

4. Schwartz, S., Zieve, L., and Watson, C. J., *J. Lab. and Clin. Med.*, 1951, v37, 843.

5. Watson, C. J., de Mello, R. Pimenta, Schwartz, S., Hawkinson, V. E., and Bossenmaier, I., *J. Lab. and Clin. Med.*, 1951, v37, 831.

6. Schwartz, S., Hawkinson, V., Cohen, S., and Watson, C. J., *J. Biol. Chem.*, 1947, v168, 133.

7. Greenberg, A. J., and Hoffbauer, F. W., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 361.

8. Legge, J. W., *Biochem. J.*, 1949, v44, 105.

9. Zieve, L., Watson, C. J., and Schwartz, S., PROC. SOC. EXP. BIOL. AND MED., in press.

10. Watson, C. J., Hawkinson, V., Schwartz, S., and Sutherland, D., *J. Clin. Invest.*, 1949, v28, 447.

11. Hoffbauer, F. W., Watson, C. J., and Schwartz, S., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 232.

12. ———, PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 238.

Received May 11, 1953. P.S.E.B.M., 1953, v83.

Urinary and Fecal Coproporphryn Excretion in Rats. II. Results in Experimental Liver Damage.* (20317)

F. W. HOFFBAUER, C. J. WATSON, AND SAMUEL SCHWARTZ.

From the Department of Medicine, University of Minnesota School of Medicine, Minneapolis.

In man an increased excretion of urinary coproporphyrin is commonly associated with liver functional impairment(1,2). A study of this phenomenon in the rat was undertaken because of the extensive use of this animal in experimental investigations of liver injury. The study was designed to determine the correlation between the degree of coproporphyrinuria and the severity of hepatic damage. In a previous study of experimental nutritional liver injury (dietary necrosis), the failure to observe increased urinary coproporphyrin was ascribed tentatively to the acuity of onset of the fatal hepatic lesion(3). Therefore, in the present study other types of acute and chronic but non-fatal forms of liver injury were investigated. These included hepatic damage induced by choline deficiency, and chemical injury induced by carbon tetrachloride vapor or by ingestion of thioacetamide. The effect of lead and of Rose Bengal plus light, known to increase urinary coproporphyrin excretion in some species, was also studied in a small group of normal rats. The fecal excretion of coproporphyrin was determined in all rats to see if an increase in the urinary/fecal ratio of this porphyrin might be a more sensitive index of liver injury than the urinary value alone.

Methods. The methods for urinary and fecal collections as well as the procedures employed for the estimation of porphyrins have been described in a previous report(4). All animals were housed in individual cages throughout the course of the experiments. All

---

* Supported in part under contracts from the Office of the Surgeon General, U. S. Army, under the sponsorship of the Commission on Liver Disease, Armed Forces Epidemiological Board, and from the Atomic Energy Commission.

TABLE I. Urinary and Fecal Coproporphyrin Excretion in Rats Maintained on a Choline Deficient Diet. 4 groups.

Rats studied	Analysis	Day of experiment										
		4	19	26–32	41	54–70	82–84	113	134–171	195	205	219
3 ♂	UCP			9	12	14	16	23	26			
	FCP			6	4	3	6	3	2			
	Body wt, g			50	54	58	64	70	78			
2 ♀	UCP			11	30	20	21					
	FCP			6	3	6						
	Body wt, g			55	60	61	60					
2 ♂	UCP	15	36	22		26	20		11	38	13	19
	FCP	10	8	8		8	7		7	14	8	9
	Body wt, g	157	153	152		172	177		163	147	142	133
4 ♀	UCP	6	16	13		12	20		17*	30*	21†	17†
	FCP	16	8	11		6	10		10*	3*	5†	5†
	Body wt, g	128	125	124		140	143		123*	110*	106†	98†
Avg ratio	UCP/FCP	1.7			4.0		4.6					

Urinary (UCP) and fecal (FCP) coproporphyrin values in μg/48 hr. Avg values listed, although separate analyses were performed on the excreta of each rat.

* Observations on 3 rats. † Observations on 2 rats.

Survival periods: Group 1—170, 184 and 198 days; Group 2—103 and 137 days; Group 3—222 and 246 days; Group 4—164, 198, 222 and 241 days.

animals received weighed amounts of the special diets employed; water was allowed *ad libitum*.

Diets employed. 1) The choline-deficient diet described by Gyorgy and Goldblatt as LVI(5) was employed. This diet contains 8% casein. Each rat received a 10 g allowance daily, with a daily supplement of 1 ml of a vit. B solution containing 20 μg of thiamin chloride, 100 μg of calcium pantothenate, 20 μg of pyridoxine, and 25 μg of riboflavin. 2) The normal diet consisted of Purina Fox-Chow. This was fed *ad libitum*. 3) The thioacetamide was added to finely-ground commercial food (Purina Fox-Chow) at a level of 0.05%. Each animal received 10 g of the diet mixture per day. *Carbon tetrachloride exposure.* Individual rats were placed in a large laboratory desiccator through which air containing approximately 50 p.p.m. of CCl_4 vapor was circulated at a rate of 3 liters per minute. In normal rats, a one-hour exposure produced a state of intoxication and, generally, a temporary hind quarter paralysis.

Results. 1. *Choline-deficient diet.* Rats maintained on choline-deficient diets develop an intense fatty infiltration of the liver. If this nutritional deficiency is long continued, a diffuse fibrosis and cirrhosis develops, as has been shown by many investigators.

Table I presents data for a group of 11 rats maintained on such a diet. The animals succumbed to the effects of the dietary deficiency at various intervals from 103 to 241 days. At autopsy all showed extensive fatty infiltration of the liver and varying degrees of fibrosis (cirrhosis). As noted in Table I, growth was markedly retarded. The weights of the 48-hour fecal samples and the volumes of the urine samples for this period were below those of well-fed normal rats. They were adequate, however, for the coproporphyrin analyses. It will be noted that the values of the FCP were consistently low throughout the entire experiment. The UCP values in the male rats were also low. Although there was a tendency toward a slight increase as the experiment progressed, the levels did not reach those shown by normal healthy rats. The females, especially those that survived longer, showed but slightly increased UCP values. In 90 control rats(4), an average urine/feces coproporphyrin ratio of 1.0 was found, average values being higher in male than in female animals. In the present study, determinations were not made prior to the institution of the choline-deficient diet. However, 23 analyses through the 32nd day showed an average UCP/FCP ratio of 1.7. From days 41-70 the average ratio in 16 analyses rose to 4.0, while the final 33 analyses from days 82-219 showed an average ratio of 4.6.

TABLE II. Influence of Choline Deficient Diets on Coproporphyrin Excretion in Rats Receiving Alcohol or Water as Drinking Fluid. 4 groups.

Rats studied	Fluid intake		Days after start of exp.								
			1	189	226	234	261	274	294–301	334–351	368–381
			–Normal diet–			Choline-deficient diet					
		Days after start of low choline diet				8	35	48	68–75	108–125	142–155
4 ♂	Water	UCP			43			21	28	57*	88‡
		FCP			66			17	14	10	10
		Body wt, g	90		335			263	208	197	207
”	10% alcohol	UCP			21			33	47	27	37‡
		FCP			33			9	14	13	12
		Body wt, g	80		264			253	219	210	180
2 ♀	Water	UCP			7			29	47	33†	38†
		FCP			41			20	16	9	11
		Body wt, g	101		237			198	182	161	161
”	10% alcohol	UCP		6		23	16		31	31	
		FCP		35		4	13		11	15	
		Body wt, g	75	193		189	175		186	170	
	Avg ratio UCP/FCP		0.5			—		2.5		4.1	

Urinary (UCP) and fecal (FCP) coproporphyrin values in $\mu g/48$ hr.
* Avg values for 3 rats. † Values for 1 rat. ‡ Avg values for 2 rats.
Survival periods after start of low choline diet: Group 1—84, 136, 193, 189 days; Group 2—108, 138, 150, 189 days; Group 3—120, 185 days; Group 4—150, 150+ days.

TABLE III. Coproporphyrin Excretion in Rats with Acute Liver Damage Due to Carbon Tetrachloride.

Rats studied	Avg wt, g	Analysis	Control values $\mu g/48$ hr	Values after exposure to carbon tetrachloride vapor, $\mu g/48$ hr 0-48 hr period	96-144 hr period
2 ♂	52	UCP	33	45	24
		FCP	51	19	31
2 ♀	103	UCP	14	20	11
		FCP	47	12	24
3 ♂	250	UCP	48	83	
		FCP	38	19	
3 ♀	187	UCP	13	24	15*
		FCP	34	—	26*
Avg ratio UCP/FCP			.7	3.0	.6

* Avg values for 2 rats only.

2. *Choline-deficient diet plus alcohol.* In an attempt to study the influence of alcohol on the UCP and FCP excretion, a group of 6 normal rats was given 10% alcohol in place of water as a drinking fluid. They appeared to tolerate this well and gained in weight while on an unrestricted normal diet (Table II). After 189 days and 226 days, UCP and FCP determinations were made. The values of the alcohol-treated male rats were somewhat lower than in those receiving water at this time. On the 226th day, all the animals were started on a low-choline diet. All animals lost weight. The FCP values were reduced just as in the first series of animals on this deficient diet, as above described. The UCP values rose somewhat as the experiment progressed, but again the levels were not striking and only occasionally exceeded those encountered in normal animals. The combined effect of alcohol and a deficient diet did not increase the UCP. Alcohol did not increase the degree of fibrosis or the severity of the cirrhosis in these animals as judged by

TABLE IV.
Coproporphyrin Excretion in Rats Maintained on a Normal Diet Plus Thioacetamide.

			Values after exp. diet (.05% thioacetamide)		
Rats studied	Analysis	Control values	35th day	77th day	Survival period, days
3 ♂	UCP	56	53	58*	42, 74, 98
	FCP	32	13	10*	
	Body wt, g	282	229	206	
2 ♀	UCP	14	33	36*	46, 77
	FCP	28	13	11*	
	Body wt, g	197	165	145	
3 ♀	UCP	14	28	37*	35, 85
	FCP	25	6	20*	
	Body wt, g	86	81	84	
1 ♂	UCP	62	36	37	78
	FCP	26	10	16	
	Body wt, g	115	118	133	
Avg ratio UCP/FCP		1.2	4.2	3.3	

Urinary (UCP) and fecal (FCP) Coproporphyrin values in μg/48 hr.
* Values for single rat.

the gross and microscopic appearance of the livers. Again the ratio of urinary to fecal coproporphyrin rose significantly on the choline-deficient diet from an average control level of 0.5 to 2.5 from days 274-301 and to 4.1 from days 334-381.

3. *Carbon tetrachloride exposure.* Ten rats were exposed to carbon tetrachloride vapor for one hour. The UCP and FCP excretion were determined before and at 0-48 and 96-144 hours after the exposure. The data shown in Table III summarize the average values for both small and large male and female rats. The period of exposure was sufficient to produce a severe but usually non-fatal form of acute liver damage. In previous studies, similar groups of rats have been sacrificed at intervals after this one-hour exposure. At 24 hours, such animals show a central form of cellular necrosis; approximately 50% of the cells of a given lobule appear necrotic. At 72 hours, recovery is well underway. If no further exposure is given, complete restitution occurs in 7 to 10 days and little evidence of the previous necrosis remains. The results of the present experiment indicate a moderate increase in the UCP values and a decrease in the FCP values during the first 48 hours, with a return toward normal in the third 48-hour period. The UCP/FCP ratio was considerably increased during the first 48-hour period. Unfortunately, laparotomies performed on the 3 adult male rats to permit liver biopsy, proved fatal. Thus the opportunity to collect urine and feces in this group during the 96-144 hour period was lost.

4. *Thioacetamide.* The effect of 0.05% thioacetamide in the diet was studied in 9 rats. This agent produces a severe form of chronic liver injury characterized by cellular necrosis, nodular regeneration, and cirrhosis(6-8). The rats succumbed at various intervals of from 35 to 98 days. Determinations of the UCP and FCP were made on the 35th and 77th days. The data are shown in Table IV. Despite the presence of severe liver injury, the only increases noted in the UCP values occurred in the females, and these were no greater than sometimes found in healthy normal female rats. Largely because of the drop in FCP values, the ratio of UCP/FCP showed a 3- to 4-fold increase. The failure of rats with various forms of acute and chronic liver damage to exhibit significant increases in coproporphyrin excretion prompted a study of the effects of other toxic agents in this species.

5. *Rose Bengal plus light.* Pimenta de Mello reported that Rose Bengal plus light exposure in rabbits resulted in a rapid and marked increase in the urinary excretion of coproporphyrin(9). This experiment was therefore repeated in rats. The exposure of a shaved area of the rat's back to unfiltered light from

TABLE V. Coproporphyrin Excretion in Rats Injected with Rose Bengal and Exposed to Ultraviolet Light.

Rat No.	Sex	Wt, g	Analysis	Control period	0-48 hr period after treatment*	Observations at 48 hr: Hematocrit, %	Observations at 48 hr: Biopsy of liver
B 37	♂	270	UCP	14	23	40.6	Normal
			FCP	32	31		
39	♂	250	UCP	24	12	43	”
			FCP	33	13		
40	♀	190	UCP	12	19	43.7	”
			FCP	14	19		
41	♀	145	UCP	16	20	40	”
			FCP	14	35		
42	♀	196	UCP	10	19	—	”
			FCP	17	19		
	Avg ratio UCP/FCP			.75	.85		

Urinary (UCP) and fecal (FCP) coproporphyrin values in μg/48 hr.

* Exposure to unfiltered light from AH-4 mercury lamp: 10 min. at distance 12 in. to 2″ × 2″ shaved area on back on animal.

TABLE VI. Coproporphyrin Excretion in Rats Following Lead Intoxication.

No. of rats	Sex	Route of administration	Analysis	Control values, μg/48 hr	Values after lead inj., μg/48 hr: 4th to 6th day	Values after lead inj., μg/48 hr: 8th to 10th day
2	♂	Intraper.*	UCP	17		207
			FCP	26		25
2	♀	” *	UCP	16		66
			FCP	18		30
6	♂	Intrav.†	UCP	53	150	
			FCP	43	18	
6	♀	” †	UCP	23	70	
			FCP	40	14	
	Avg ratio UCP/FCP			0.86	6.4	

* 10 mg lead acetate/100 g body wt.

† 2 ” ” ” ” ” ”.

an AH-4 mercury lamp immediately followed the intravenous injection of Rose Bengal in a dose of 10 mg per 100 g body weight. Results obtained in 5 animals are summarized in Table V. As noted, no appreciable increase was found in the excretion of UCP or FCP, or in their ratio. It is noteworthy, too, that unlike Pimenta de Mello's rabbits, these rats showed no evidence of a significant hemolytic reaction. No pathological changes were noted in their livers at the end of a 48-hour period.

6. *Lead.* Lead acetate was administered to rats both by intravenous and intraperitoneal routes. The data obtained are presented in Table VI. The intraperitoneal administration produced a severe chemical peritonitis. The liver was covered by a heavy exudate; despite this, the parenchymal cells appeared normal on histologic examination. The intravenous injection of 0.4 or 0.5 ml of a 1% solution of the lead salt was well tolerated by 6 male and 6 female rats. In 2 additional animals similarly treated an hepatic lobectomy and a unilateral nephrectomy was performed at 24 hours and the animals were sacrificed at the end of 72 hours. A study of the liver sections revealed a minimal degree of fatty infiltration at 24 hours; this had disappeared at 72 hours. The kidneys appeared normal on microscopic examination. As noted in Table VI, all animals responded by a marked increase in the UCP. The values for the FCP

remained within the normal range.

Discussion. Despite the production of histologically demonstrable hepatic lesions, it is evident that choline deficiency, carbon tetrachloride, and thioacetamide produced little if any increase in the urinary excretion of coproporphyrin in the rats studied. This is quite unlike results observed in humans with various types of liver disease(1,2). These rats did, however, show significant increases in the average ratios of urinary to fecal coproporphyrin excretion, though a considerable amount of individual variation in values was observed. This observation tends to confirm the suggestion(10,11) that elevation of this ratio may be of greater significance in liver disease than is either the urinary coproporphyrin or fecal coproporphyrin value alone. Further confirmation of this is offered in paper III of this series(12).

As regards the effect of lead poisoning, at least, it seems that the porphyrin excretion of the rat is similar to that of other species studied. On the other hand, the response of the rat to Rose Bengal and light is quite unlike that reported for the rabbit.

No attempt has been made to investigate the effect of hepatotoxic agents on the excretion of uroporphyrin in rats since the studies reported here were completed before the demonstration that uro-type porphyrins are normally found in rat excreta(4).

Summary and conclusions. 1. Despite the presence of severe liver involvement, no consistently significant increases were noted in the excretion of urinary or fecal coproporphyrin in rats fed a choline-deficient diet or thioacetamide. Average 3- to 4-fold increases, however, were observed in the ratio of urinary to fecal coproporphyrin. 2. Essentially similar results were obtained in choline-deficient rats maintained on either alcohol or water as drinking fluid. 3. Transitory increases were found in the urinary coproporphyrin and the urinary/fecal coproporphyrin ratio following acute exposure to carbon tetrachloride vapor. 4. The combination of Rose Bengal and ultraviolet irradiation failed to produce any increase in urinary porphyrin excretion in rats. 5. The injection of lead in rats produced a striking increase in the excretion of urinary coproporphyrin.

The authors gratefully acknowledge the valuable assistance of Miss Bernadine Wittenburg and Mr. Michael Keprios in this study.

---

1. Watson, C. J., and Larson, E. A., *Physiol. Rev.*, 1947, v27, 478.

2. Sutherland, D., and Watson, C. J., *J. Lab. and Clin. Med.*, 1951, v37, 29.

3. Greenberg, A. J., and Hoffbauer, F. W., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 361.

4. Hoffbauer, F. W., Watson, C. J., and Schwartz, S., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 228.

5. Gyorgy, P., and Goldblatt, H., *J. Exp. Med.*, 1949, v89, 245.

6. Ambrose, A. M., De Eds, F., and Rather, L. J., *J. Industr. Hyg. and Tox.*, 1949, v31, 158.

7. Fitzhugh, O. G., and Nelson, A. A., *Science*, 1948, v108, 626.

8. Rather, L. J., *Bull. Johns Hopkins Hosp.*, 1951, v88, 38.

9. Pimenta de Mello, R., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 292.

10. Localio, S. A., Schwartz, M. S., and Gannon, C., *J. Clin. Invest.*, 1941, v20, 7.

11. Nesbitt, S., and Snell, A. M., *Arch. Int. Med.*, 1942, v69, 573.

12. Hoffbauer, F. W., Watson, C. J., and Schwartz, S., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 238.

Received May 11, 1953. P.S.E.B.M., 1953, v83.

Urinary and Fecal Coproporphyrin Excretion in Rats. III. Excretion of Injected Coproporphyrin.* (20318)

F. W. Hoffbauer, C. J. Watson, and Samuel Schwartz.

From the Department of Medicine, University of Minnesota School of Medicine, Minneapolis.

Previous papers have dealt with the urinary (UCP) and fecal (FCP) excretion of coproporphyrin in normal rats(1) and in rats subjected to various forms of nutritional and chemical liver injury(2). The latter group included especially rats on a choline-deficient diet and those treated with thioacetamide or with carbon tetrachloride vapor. Among these rats, only those treated with carbon tetrachloride showed even a moderate increase in coproporphyrinuria. All, however, showed considerable elevations of the ratio of urinary to fecal coproporphyrin.

Since elevation of this ratio has been reported(3,4) to correlate better with liver dysfunction than does either value alone, studies were carried out to determine the relationship between liver function and the pathway of excretion of intravenously administered coproporphyrin. The present report is concerned with an analysis of such studies in normal rats and in rats with experimentally induced liver damage. An attempt was also made to determine the pathway of excretion of injected coproporphyrin after ligation of the common bile duct and after the creation of an external biliary fistula.

Methods. 1) *Administration of Coproporphyrin.* The 48-hour UCP and FCP excretion were first determined. A solution containing 250 to 500 μg of coproporphyrin isomer I or III was then injected into a foot vein, and the UCP and FCP determinations were repeated during the following 48 hours. The coproporphyrin used was crystallized as the methyl ester, and saponified in a few ml of 7.5 N hydrochloric acid. Following neutralization of the acid with sodium acetate, the porphyrin was extracted with ethyl acetate. The latter was washed repeatedly with water and vacuum distilled to dryness. The porphyrin was dissolved either in a 0.16 M sodium carbonate solution or in a 5% sodium bicarbonate solution just before its administration in a total volume of about 1.5 ml. 2) *Production of liver damage.* The procedures used for the production of liver damage by carbon tetrachloride and by a choline-deficient diet have been described previously(2). 3) *Production of biliary obstruction and biliary fistulae.* Obstruction of the common bile duct was performed by placing double ligatures of No. 5 silk as close to the hilus as possible in order to confine the resulting marked dilatation of the bile ducts to the intrahepatic portion of the system. Creation of an external biliary fistula was accomplished at laparotomy by inserting a plastic tube (internal diameter 0.11 inch, external diameter 0.23 inch) into the common bile duct and securing it in place with silk sutures. The tubing was passed through the peritoneal cavity and by a subcutaneous route to the outside near the base of the skull. The tube was then passed through a small cork. A finger cot was fastened about the cork and formed a reservoir to collect the bile. The finger cot was held in place on the animal's back by means of a small plastic cover. Bile was aspirated from the sack, usually every 6 hours, to prevent any rise in pressure. Rats tolerate this procedure well and are free to move about in their cages. 4) *Analytical methods.* Methods employed have been described in a previous communication(1).

Results. 1) *Fate of injected coproporphyrin.* When a solution of coproporphyrin (500 μg of isomer III in 1.5 ml of 5% sodium bicarbonate) is injected intravenously into an anesthetized normal animal, the bile duct very quickly exhibits an intense red fluorescence when viewed in ultraviolet light. The intensity and rapidity of the development of fluorescence is very similar to that seen after the intravenous injection of fluorescein. In

---

* Supported in part under contracts from the Office of the Surgeon General, U. S. Army, under sponsorship of the Commission on Liver Disease, Armed Forces Epidemiological Board, and from the Atomic Energy Commission.

TABLE I. Porphyrin Excretion (μg/48 Hr) in Rats with Altered Biliary Outflow.

No. of rats	Analyses		Values		Procedures
			Pre-op.	Post-op.	
2	Urine	C*	36.6	23.1	External biliary fistula (plastic tube); 500 μg coproporphyrin III intrav.
	Feces	C	41.8	18.9	
		P	252.5	66.5	
	Bile	C		259.6	
2	Urine	C	54.5	25.2	External biliary fistula (plastic tube); 500 μg coproporphyrin via stomach tube
	Feces	C	22.0	162.3	
		P	110.0	105.8	
	Bile	C		27.6	
	Intestinal content	C		119.7	
		P		30.0	
1	Urine	C	31.2	30.9	500 μg coproporphyrin III, intrav.
	Feces	C	32.0	302.4	
		P	130.0	147.2	
	Urine	C		281.4	Ligated common bile duct; 500 μg coproporphyrin III intrav.
	Feces	C		16.2	
		P		56.0	
1	Urine	C	41.6	43.4	Ligated common bile duct; (no coproporphyrin given)
	Feces	C	55.0	13.6	
		P	220.0	50.0	

C = Copro; P = Proto.

one such experiment, the bile was collected via a plastic tube inserted in the duct; the animal was kept in a state of anesthesia with ether. In 2 hours, 177 μg of coproporphyrin were recovered in the 2.1 ml of bile obtained. The concentration of coproporphyrin in the blood at the end of the 2-hour period was 3.8 μg per ml.

The changes in the urinary and fecal excretion of coproporphyrin and protoporphyrin in six male rats with various alterations of the bile duct system, are shown in Table I. These rats each weighed approximately 250 g. It is to be noted that when the bile was prevented from reaching the intestine, the coproporphyrin of the feces decreased. Since the collections were made in the immediate 48-hour postoperative period, the presence of some coproporphyrin is to be expected, because the first portion of the feces excreted represents material present in the intestinal tract at the time of surgery. When coproporphyrin is injected intravenously, the preferential route of excretion appears to be by way of the bile as judged by the recovery of the porphyrin in the bile in the case of an external biliary fistula or in the feces where the bile duct is patent. When the bile outflow is obstructed, the pigment appears in the urine. The absorption of the material from the gastrointestinal tract seems to be minimal since oral administration did not result in an increase of porphyrin in either the urine or bile. (Table I.) No more than trace amounts of protoporphyrin were present in the bile and urine samples.

2) *Effect of acute carbon tetrachloride exposure.* The excretory patterns of intravenously administered coproporphyrin I in 6 normal male and 6 normal female rats are presented in Table II. After the control values for the UCP and FCP were obtained, each rat received 500 μg of coproporphyrin I intravenously. This resulted in no appreciable increase in the UCP, while the FCP increased to a considerable extent. Approximately one-half of the injected coproporphyrin could be accounted for by the increase in the FCP.

After the above determinations were made, the rats were exposed for one hour to carbon tetrachloride vapor. Twenty-four hours later, when liver damage was at its height, coproporphyrin I was again administered. As shown in Table II the rat now exhibited an increase in the UCP equal to that of the FCP.

A similar study, employing coproporphyrin III, was conducted in another group of rats. The results are shown in Table III. Prior to

TABLE II. Excretion of Injected Coproporphyrin I by Rats before and after Production of Liver Damage by Carbon Tetrachloride.

No. of rats studied	Analysis*		Control values	Values after 500 µg coproporphyrin I, I.V. Before liver damage	 After liver damage
6 ♂	UCP	Range	30–53	21– 54	68–372
		Avg	46	41	152
	FCP	Range	23–40	144–406	39–224
		Avg	32	306	152
6 ♀	UCP	Range	12–30	19– 40	38–273
		Avg	20	30	164
	FCP	Range	19–64	204–379	110–236
		Avg	43	310	164

* Urinary (UCP) and fecal (FCP) coproporphyrin values in µg/48 hr.

TABLE III. Excretion of Injected Coproporphyrin III by Rats before and after Production of Liver Damage by Carbon Tetrachloride.

No. of rats studied	Analysis*		Control values	Values after 500 µg coproporphyrin I, I.V. Before liver damage	 After liver damage
5 ♂	UCP	Range	31–54	27– 58	51– 87
		Avg	45	46	72
	FCP	Range	16–64	318–480	133–252
		Avg	29	379	175
6 ♀	UCP	Range	11–22	15– 18	42–184
		Avg	16	17	122
	FCP	Range	5–42	215–530	21–208
		Avg	26	292	73

* Urinary (UCP) and fecal (FCP) coproporphyrin values in µg/48 hr.

the production of liver damage, the intravenous injection of 500 µg of coproporphyrin III again resulted in an increase of only the FCP. Twenty-four hours after carbon tetrachloride exposure, when liver damage was maximal, the study was repeated. The UCP values then increased slightly in the male but to a considerable extent in most of the females. As in the case of the isomer I study, less than half of the injected material subsequently appeared in the urine or feces.

3) *Effect of chronic choline deficiency.* The fate of injected coproporphyrin (isomer type III) was also studied in 6 female rats in which fatty liver and early cirrhosis existed. This form of liver damage was produced by maintaining the rats for 117 days on a choline-deficient diet. Because of the animals' failure to grow appreciably on such a diet, their average body weight was only 90 g at the time of the experiment. Therefore, only 250 µg of the porphyrin was administered. As had been noted in previous studies(2), the control UCP and FCP values were low in these animals despite the presence of severe liver damage (Table IV). No appreciable increase in either the UCP or the FCP was observed after the injection of coproporphyrin despite the fact that two successive 48-hour collections were made.

4) *Effect of post-hemorrhagic anemia.* Because rats on a choline-deficient diet exhibit anemia, it was felt that the disappearance of essentially all the injected coproporphyrin in the above-treated rats might in some way be associated with the anemia. Four rats were therefore bled after control data for the UCP and FCP were obtained. A coproporphyrin excretion study after injection of 500 µg of isomer type III was made at a time when the hemoglobin and erythrocyte counts were reduced and a mild reticulocytosis existed. As shown in Table V, results were similar to those observed in normal rats (Tables II and III); nearly half of the injected coproporphyrin was excreted in the feces.

Discussion. It would appear from these studies that about half of the intravenously administered coproporphyrin is excreted in the feces in normal rats, while essentially none is excreted in the urine. Since studies have shown a rapid increase of porphyrin in the bile of rats treated in this way, it seems certain that the injected porphyrin is excreted by the liver into the intestinal tract. The data pre-

TABLE IV. Excretion of Injected Coproporphyrin III by Rats with Fatty Livers and Early Cirrhosis (Choline Deficient Diet, 117 Days).

No. of rats studied	Analysis*		Control values	Values after 250 µg CP III, I.V. 1st 48 hr	 2nd 48 hr
6 ♀	UCP	Range	9–28	7–29	8–34
		Avg	16	19	20
	FCP	Range	1–10	10–47	7–53
		Avg	6	22	39

* Urinary (UCP) and fecal (FCP) coproporphyrin values in µg/48 hr.

TABLE V. Excretion of Injected Coproporphyrin III by Rats with Acute Post-Hemorrhagic Anemia.

						Hematological data 24 hr after bleeding (3 ml blood loss)		
Rat No.	Sex	Wt, g	Analysis*	Control values before bleeding	Values after 500 μg CP III, I.V., after bleeding	Hb., g/100 ml	RBC	Retic., %
D 33	♀	130	UCP	44	26	8.6	4.2×10^6	3.0
			FCP	25	230			
34	♀	120	UCP	42	20	9.4	3.86 × ”	3.8
			FCP	45	240			
37	♂	130	UCP	61	88	10.2	4.18 × ”	3.4
			FCP	47	296			
38	♂	130	UCP	70	77	10.4	3.62 × ”	2.4
			FCP	34	180			

* Urinary (UCP) and fecal (FCP) coproporphyrin values in μg/48 hr.

sented suggest that the ability of the functionally damaged liver to excrete coproporphyrin is impaired. This leads to reduced fecal values and to increased urinary excretion of the porphyrin.

The above findings and interpretation are consistent with the previous report of increased ratios of UCP/FCP excretion in rats with various forms of experimental liver damage(2), as well as with similar reports in humans with liver disease(3,4). However, they leave several pertinent questions unanswered: 1) What is the fate of that portion of the injected porphyrin (approximately 50 per cent) not accounted for in the excreta? 2) Why was a still larger fraction unaccounted for in the choline-deficient rats? 3) How can the presence of marked coproporphyrinuria with normal fecal coproporphyrin values in lead poisoning(2) be harmonized with the concept of diversion by altered liver function? 4) Why do normal rabbits that are injected intravenously with 500 to 1000 μg of coproporphyrin show no subsequent increase in either urinary or fecal coproporphyrin values(5)?

These questions might perhaps be answered if we knew something of the further metabolism of coproporphyrin in the body, *i.e.*, its possible position as a precursor in the synthesis of heme compounds. This possibility is being investigated by the administration of C^{14}-labelled porphyrins. Another possibility which must be considered is that the coproporphyrin occurring naturally in blood and tissues differs significantly from the injected material. Previous studies(6,1) have shown that approximately half of the freshly-passed urinary coproporphyrin is in the form of a non-fluorescing precursor which is converted to coproporphyrin by iodine, light, etc. It seems highly probable that the coproporphyrin in the body is also present in the form of some such precursor. Furthermore, since porphyrins combine readily with proteins, it may be that the porphyrin-protein complex of the native porphyrin is different from that of the injected porphyrin. The excretion pattern of the two might therefore be different.

Studies of experimental porphyria in rabbits(7-9) as well as studies in humans(10,11) have indicated two major sources of the excreted porphyrin, namely the developing red cells of the bone marrow and the liver. These studies, as well as those of patients exhibiting increased blood regeneration, suggest that the source of the porphyrin has no primary relationship to the excretory pattern observed. Thus, for example, the bone marrow is primarily involved in lead poisoning and erythropoietic (congenital) porphyria. Nevertheless, in the former condition coproporphyrin is excreted almost entirely in the urine, while in the latter, it appears chiefly in the feces.

The absence of increased urinary porphyrin excretion following the "oral" administration of coproporphyrin is in agreement with similar observations by Larson and Watson in human subjects(12). In view of the fecal recovery of only half of the administered material, however, the intestinal absorption of coproporphyrin is still possible. The use of C^{14}-labelled porphyrin in such an experiment should yield decisive information in this regard.

Summary and conclusions. 1. Studies have been made of the urinary and fecal excretion of coproporphyrins I and III administered intravenously to rats. In the normal animal, no increase in urinary excretion occurred; approximately one-half of the injected material was excreted in the feces. 2. Coproporphyrin administered intravenously to rats was rapidly excreted in the bile via an external biliary fistula, or in the urine when complete biliary obstruction existed. Orally administered coproporphyrin did not appear to be absorbed, and approximately half was recovered from the feces. Regardless of the route of administration, only half of the coproporphyrin could be recovered. 3. In acute liver injury due to carbon tetrachloride, intravenous administration of coproporphyrin resulted in an increased urinary porphyrin excretion, with a relative reduction in the amount excreted in the feces. 4. In rats with chronic liver injury due to a choline-deficient diet virtually none of the injected porphyrin could be recovered in either urine or feces. The reason for this is unknown. 5. Rats made anemic by acute bleeding exhibited normal (*i.e.,* increased) fecal excretion of coproporphyrin III, following its intravenous administration.

The authors gratefully acknowledge the valuable assistance of Miss Bernadine Wittenburg and Mr. Michael Keprios in this study.

---

1. Hoffbauer, F. W., Watson, C. J., and Schwartz, S., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 228.

2. ———, PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 232.

3. Localio, S. A., Schwartz, M. S., and Gannon, C., *J. Clin. Invest.,* 1941, v20, 7.

4. Nesbitt, S., and Snell, A. M., *Arch. Int. Med.,* 1942, v69, 573.

5. Schwartz, S., and Zagaria, R., U. S. Atomic Energy Commission, MDDC-504, (1946). *Studies of porphyrin metabolism.* II. *The effect of metals on porphyrin excretion.*

6. Watson, C. J., de Mello, R. P., Schwartz, S., Hawkinson, V. E., and Bossenmaier, I., *J. Lab. and Clin. Med.,* 1951, v37, 831.

7. Schmid, R., Hanson, B., and Schwartz, S., PROC. SOC. EXP. BIOL. AND MED., 1952, v79, 459.

8. Schwartz, S., Keprios, M., and Schmid, R., PROC. SOC. EXP. BIOL. AND MED., 1952, v79, 463.

9. Schmid, R., and Schwartz, S., PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 685.

10. Watson, C. J., Lowry, P. T., Schmid, R., Hawkinson, V. E., and Schwartz, S., *Trans. Assoc. Am. Phys.,* 1951, v64, 345.

11. Watson, C. J., Chapt. 19 in Duncan's *Diseases of Metabolism,* 3rd Ed., Saunders Pub. Co., Phila., 1952.

12. Larson, E. A., and Watson, C. J., *J. Clin. Invest.,* 1949, v28, 452.

Received May 11, 1953. P.S.E.B.M., 1953, v83.

Proteolytic, Hypotensive and Thromboplastic Properties of Hypochlorite Treated Trypsin.* (20319)

DONALD E. BOWMAN.

From the Department of Biochemistry and Pharmacology, Indiana University School of Medicine, Indianapolis.

It is well known that the parenteral administration of trypsin induces a reaction similar to that of an antigen-antibody response with a sharp fall in blood pressure resulting from intravenous administration(1). That the inhibition of the proteolytic activity of the enzyme does not necessarily diminish its hypotensive effect was pointed out earlier(2). It was also reported that iodination(3,4) and treatment with formaldehyde(2) serve to decrease the hypotensive activity of trypsin more than its proteolytic or thromboplastic effects. Since the proteolytic and hypotensive properties are separable more advantageous means of accomplishing this separation were

---

* These studies were aided by a contract between the Office of Naval Research, Department of the Navy, and Indiana University.

sought. This was done, in part, with the view of attempting to improve the enzyme as a possible agent for the elimination of protein debris and the digestion of fibrin *in vivo*.

The present report deals with proteolytic, hypotensive and thromboplastic properties of trypsin treated with sodium hypochlorite(5). In a subsequent report its use in combating fibrosis and the promotion of healing will be described.

Methods. Sodium hypochlorite was added to a 0.4% solution of commercial trypsin. After standing 18 hours at 5°C the solution was dialysed in distilled water at 5°C for 24 hours and either lyophilized or precipitated with 80% ethyl alcohol, washed in absolute alcohol and dried *in vacuo*. In order to minimize denaturation of crystalline enzymes 90 ml of a cold solution of NaOCl was added to 0.25 g of the enzyme in 10 ml of cold water. After 5 hours at 5°C the preparation was dialysed at the same temperature in a mechanical dialyser for 4 hours and lyophilized. After dialysis tests for available chlorine were negative. Proteolytic activity was usually estimated by the procedure described by Kunitz(6) based on the increase in optical density of a filtrate of a casein digestion mixture. Changes in proteolytic activity were estimated by determining the amount of the treated enzyme required to cause the same change in optical density as a given amount of untreated trypsin with the same solution of casein. The relative proteolytic activity of acetone treated plasma due to added protease was estimated by an adaptation of a method of Schmitz(7). One ml of plasma was precipitated in 95% acetone. The precipitate was washed with acetone, centrifuged and dried in vacuum. The precipitate was thoroughly ground with the aid of a glass rod in the centrifuge tube and one ml of 0.08 M phosphate buffer, pH 7.4, containing 10 mg of merthiolate per 100 ml was added. After standing at 5°C for one hour with the rod remaining in the tube the contents were thoroughly mixed and 9 ml of buffer added. The tubes were stoppered and incubated at 38°C for 48 hours. After precipitating with 10% trichloroacetic acid the non-protein nitrogen of the filtrate was estimated by the micro-Kjeldahl procedure. Values given were corrected for the original non-protein nitrogen and that due to the activity of the partially activated natural protease of the plasma. All glassware coming into contact with the blood or plasma was siliconed. Hypotensive activity was estimated employing dogs and the usual methods of carotid cannulation and recording. After the repeated initial injection of a standard fixed dose of 0.1 to 0.5 mg of untreated trypsin per kg a reasonably reproducible response was obtained in a given dog with a return to the control blood pressure level following each injection. Changes in hypotensive effect were determined by observing the amount of the treated enzyme required to produce approximately the same degree and duration of decreased blood pressure as a standard dose of untreated trypsin injected before and after the treated enzyme. In observing coagulation time plasma containing 1.34 mg of sodium oxalate per ml was diluted with 2 ml of saline. To 0.5 ml of diluted plasma at 38°C was added 0.5 ml of a solution prepared by adding 2 ml of saline or enzyme solution to 5 ml of 0.222% $CaCl_2$. The tubes were gently tilted 40 times per minute at 38°C until gel formation was observed and verified by turning the tube horizontally. The plasma was obtained from blood drawn from a cannulated carotid artery discarding the first blood obtained. After centrifuging at 15,000 rpm for 30 minutes at 18°C the plasma was drawn off to within one cm of the cells. All glassware was siliconed. The term trypsin refers to commercial trypsin unless otherwise indicated. Rabbits were used in most of this work because of the marked susceptibility of this species to the thromboplastic effect of trypsin and trypsin embolism.

Results. Suitable amounts of hypochlorite markedly reduce the hypotensive activity of commercial trypsin while ability to digest casein is actually increased (Table I). With larger amounts of hypochlorite proteolytic activity is also diminished. In a large series of preparations such changes have been relatively reproducible. In spite of efforts to minimize denaturation of crystalline chymotrypsin and crystalline trypsin marked loss o

TABLE I. Influence of Sodium Hypochlorite on Hypotensive and Proteolytic* Activities of Trypsin and Chymotrypsin.

g NaOCl/ g enzyme	% of control activity					
	Commercial trypsin		Crystalline trypsin		Crystalline chymotrypsin	
	Hypotensive	Proteolytic	Hypotensive	Proteolytic	Hypotensive	Proteolytic
.007			50	49	50	60
.014			33	39	22	45
.028	5	143	16	19	12	36
.055	5	150	†	11	11	17
.11	4	145	†		†	
.22	3	108	†	Inactive	†	Inactive
.44	.75	25				

* Casein digestion.

† Low solubility prevented preparation of solutions practical for inj.

solubility with probable serious degradation resulted from hypochlorite treatment. However, to some extent crystalline chymotrypsin undergoes relative changes similar to those of commercial trypsin. Approximately equal losses in hypotensive and proteolytic activities occurred in crystalline trypsin. Effort is being made to further minimize the possible obscuring effect of denaturation.

When added to plasma *in vitro* hypochlorite treated trypsin imparts greater proteolytic activity to the acetone precipitate of the plasma than does untreated trypsin (Table III). The activity of such an acetone precipitate has been attributed to free protease of the plasma by some investigators; however, proteolytic activity of the precipitate is routinely observed with plasma having a net inhibitor effect prior to precipitation. We have used acetone precipitation as a reasonably reproducible means of revealing content of readily activated protease. Subsequent further treatment with 10% trichloroacetic acid, chloroform and streptokinase in this order results in progressively greater activity. That the mechanism of the action of hypochlorite may be to a large extent oxidative in nature is indicated by the differential effect of hydrogen peroxide on the proteolytic and hypotensive activities of commercial trypsin (Table II). Treatment with peroxide was similar to that with hypochlorite.

Spontaneous changes in trypsin which is allowed to stand in aqueous solution for 5 to 25 hours at 5 to 37°C also involve greater loss of hypotensive activity. In some cases the latter may fall to 20% of the original level

TABLE II. Influence of Hydrogen Peroxide on Hypotensive and Proteolytic Activities of Commercial Trypsin.

g H_2O_2/g trypsin	% of control activity	
	Hypotensive	Proteolytic
.075	33	162
.15	29	144
.225	20	137
.33	15	130

TABLE III. Increase in Proteolytic Activity of Acetone Treated Rabbit Plasma Following Addition of Trypsin and Hypochlorite Treated* Trypsin *in Vitro*.

Material added	Amt added to plasma, mg/ml	Increase in N.P.N. in digestion mixture due to activity of added enzyme, mg/100 ml
Trypsin, untreated	.10	7.4
	.20	15.9
	.08	10.3
Trypsin, hypochlorite treated	.10	20.1
	.20	27.3

* .11 g NaOCl/g trypsin.

TABLE IV. Thromboplastic Activity of Hypochlorite Treated Commercial Trypsin *In Vitro*.

mg enzyme/ ml plasma	% of control coagulation time
.00025	85
.001	68
.002	50
.012	22
.020	15
.072	7.4
.010	6.7
.020	6.5
.024	5
.048	4
.072	6
.096	5
1.3	Incoagulable

TABLE V. Influence of Heparin on Trypsin Embolism in Rabbits.

No. of animals	Substance inj.*	No.	No. with gross emboli	Amt of trypsin inj., mg/kg Avg total	Max	Min
		Fatal cases				
44	Trypsin	40	38	8	19	2
20	Cl-trypsin	14	14	18	31.5	3
20	Cl-trypsin and heparin	0				
		Sacrificed cases				
44	Trypsin	4	0	6	6	6
20	Cl-trypsin	6	3	23.3	24	20
20	Cl-trypsin and heparin	20	0	25	25	25

* Each substance inj. at rate of 1 mg/kg/15 min.

while the proteolytic activity is altered very little; however, such changes are unpredictable and difficult to reproduce.

Trypsin treated with 0.11 g NaOCl per g of enzyme has marked thromboplastic activity when added to plasma *in vitro* in suitable amounts (Table IV). An excess interferes with blood coagulation through digestion of fibrinogen as indicated by the subsequent addition of thrombin. Except in concentrations causing maximal clot acceleration the coagulation time is approximately equivalent to that obtained with 50% more untreated trypsin.

The coagulation of plasma obtained from rabbits after injecting hypochlorite treated trypsin is also accelerated. In a typical case 5, 60, and 180 minutes after injecting 2.5 mg/kg of plasma coagulation times were respectively 22, 33, and 49% of the control time. However, as with untreated trypsin intravenous administration involves considerable risk of fatal embolism in rabbits. Such embolism is prevented by the simultaneous injection of an equal amount of heparin (Table V).

As estimated by the non-protein nitrogen procedure the injection of 25 mg/kg of treated trypsin at the rate of 1 mg/kg every 15 minutes with an equal amount of heparin resulted in an increase in proteolytic activity of acetone precipitated plasma which was slightly greater than that observed upon adding 0.1 mg of the treated enzyme alone to 1 ml of plasma *in vitro*.

Heparin does not alter the hypotensive effect of hypochlorite treated trypsin and in a 1:1 ratio, at pH 7.4, does not diminish the digestion of casein. However, with the same proportions and pH, heparin decreased the proteolytic activity of acetone precipitated plasma containing 0.1 mg of treated trypsin per ml to about half of the non-protein nitrogen index obtained without heparin.

Even with the injection of 25 mg of hypochlorite treated trypsin in rabbits at the rate of 1 mg/kg every 15 minutes the plasma trypsin inhibitor level remains essentially unchanged.

Summary. Treatment of commercial trypsin with suitable amounts of sodium hypochlorite greatly diminishes the hypotensive effect of the enzyme while its proteolytic activity is increased by approximately 50%. Similar changes result from treatment with hydrogen peroxide. Hypochlorite treated trypsin possesses a marked thromboplastic effect. Heparin prevents fatal embolism which otherwise results in a high percentage of cases following the intravenous injection of treated trypsin in rabbits. An increase in the readily activated protease fraction of plasma is observed after adding the treated enzyme to plasma *in vitro* and after injecting it intravenously.

---

1. Dragstedt, C. A., and Wells, J. A., *Quart. Bull.*, Northwestern Univ. Med. School, 1944, v18, 104.

2. Bowman, D. E., PROC. SOC. EXP. BIOL. AND MED., 1948, v68, 358.

3. ———, PROC. SOC. EXP. BIOL. AND MED., 1946, v63, 408.

4. ———, PROC. SOC. EXP. BIOL. AND MED., 1947, v66, 262.

5. ———, *Fed. Proc.*, 1951, v10, 165.

6. Kunitz, M., *J. Gen. Physiol.*, 1947, v30, 291.

7. Schmitz, A., *Z. Physiol. Chem.*, 1937, v250, 37.

Received May 11, 1953. P.S.E.B.M., 1953, v83.

Biliary Excretion of Cholic Acid and Cholesterol in Hyper-, Hypo-, and Euthyroid Rats.[*] (20320)

James C. Thompson and Harry M. Vars.

From the Harrison Department of Surgical Research, Schools of Medicine, University of Pennsylvania, Philadelphia.

Previous studies from this laboratory(1,2) have indicated that cholic acid and cholesterol excretion in the bile can be followed serially for a period of over 14 days. The ratios of excretion of these constituents remained quite constant. The isolation of labeled cholic acid from the bile of animals previously given deuterium-labeled(3) or tritium-labeled(4) cholesterol is evidence for the consideration of cholic acid as a metabolic degradation product of cholesterol. Because of the well-known effect of changes in thyroid activity on blood cholesterol(5), it was of interest to ascertain the effects of altered states of thyroid activity on the biliary output of cholic acid and cholesterol.

Recent studies on the relation of thyroid function to cholesterol by Rosenman, Byers, *et al.*(6-10) have led these authors to conclude that the metabolism of cholesterol is increased in hyperthyroidism and decreased in hypothyroidism. In their experiments the biliary excretion of cholesterol was determined in experimental hyper- and hypothyroidism (8-10) upon bile collected for 24 hours following cannulation of the bile duct. During this period we have found the amount of bile secreted and its composition to be disturbed and erratic. The present work details the effects of altered states of thyroid activity on the serial analysis of the cholic acid-cholesterol inter-relationship in bile collected over a period extensive enough to allow hepatic physiology time to recover from operative trauma.

Experimental. All animals used in this work were young male Wistar rats ranging in weight from 220-260 g. They all were fed a standard synthetic diet containing 3% fat and 18% casein(11)[†] throughout the experimental period. Following cannulation of the bile duct, animals were given 1% NaCl to drink *ad libitum.* The animals were divided into 3 main groups. The control (euthyroid) group was treated as outlined. Hyperthyroid rats were prepared by feeding 100 mg thyroid (Parke, Davis USP) per day for 10-15 days preceding bile duct cannulation. Following operation, the thyroid was given as 0.33% of the diet. These animals failed to gain weight in spite of increased food intake and had an increased pulse rate and generalized tremors. Hypothyroid rats were prepared by administering propyl thiouracil,[‡] as 0.5% of the diet, for 18-21 days preoperatively and were maintained on the same dosage following cannulation. These animals lost little weight in spite of a markedly diminished food consumption.

Cannulation of the common bile duct was accomplished by following the technic described by Fisher and Vars(1). In this, a polyethylene tube (PE 50) with an internal diameter of 0.023 inch, is inserted into the duct at the junction of its proximal and middle thirds. The tube is brought through the body wall and threaded subcutaneously to emerge on the dorsal mid-line at the base of the tail, and be inserted into a latex bag. This bag is changed every 24 hours, the bile volume measured and aliquots taken for analysis of cholic acid by method of Irvin, Johnston, and Kopala(12) and of cholesterol by method of Foldes and Wilson(13).

Results. The data presented were obtained

---

[*] This work was done (in part) under Contract between the Department of the Army and the University of Pennsylvania.

		%
[†] Diet—	Casein	18
	Sucrose	73
	Cod liver oil	3
	Celluflour	2
	Jones salt Mixture No. 12	4

This diet was supplemented with menadione and adequate B vitamins.

[‡] Furnished through the courtesy of Lederle, Inc.

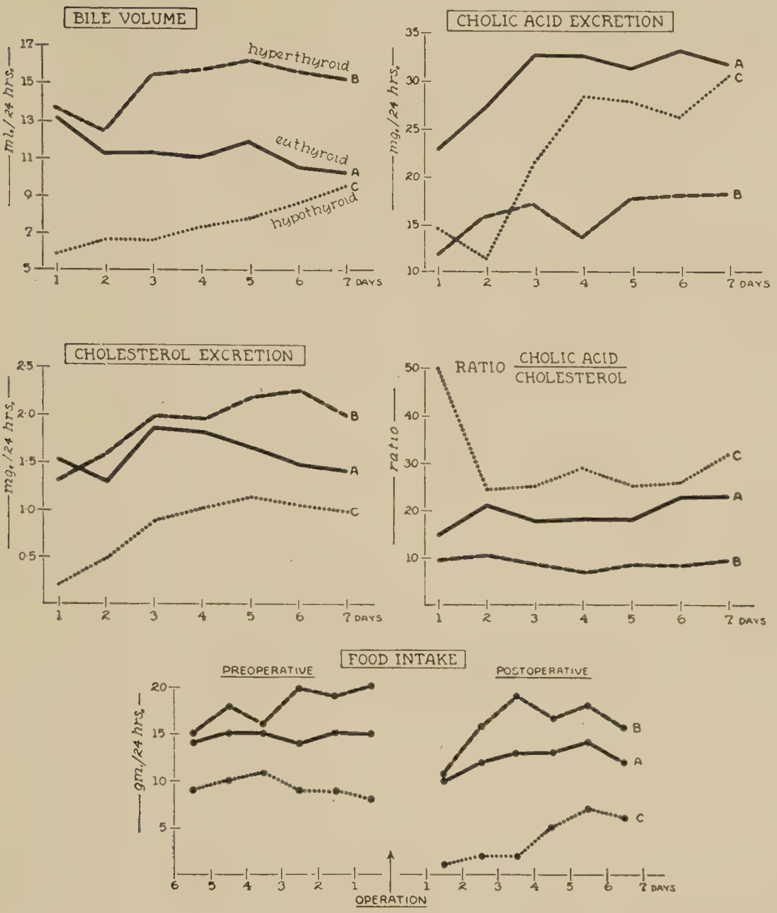


FIG. 1. Average daily biliary data, and food intake of hyper-, hypo-, and euthyroid rats.

over a bile collection period of 7 days. No animal was included in the series that was not stabilized as to food and water intake and volume of bile produced for a period of at least one day in excess of the 7 reported here.

The results are presented in graphic form in Fig. 1. The animals are divided into Group A, euthyroid control; Group B, hyperthyroid; and Group C, hypothyroid. Each group represents 10 animals. The average results were as follows (all averages except those for food intake are for the 7-day period postoperatively):

1. Volume—A:11.3 ml, B:14.9 ml, C:7.5 ml.

2. Cholic acid excretion — A:30.3 mg, B:16.0 mg, C:23.0 mg.

3. Cholesterol excretion — A:1.58 mg, B:1.89 mg, C:0.82 mg.

4. Cholic acid/cholesterol ratio—A:19.2, B:8.5, C:28.1.

5. Food intake—The animals failed to eat on the day of operation. Average figures reported are for 6 days preceding operation, and for second to seventh day postoperative. Preoperative—A:15 g, B:18 g, C:9 g. Postoperative—A:12 g, B:16 g, C:4 g.

As can be seen in Fig. 1, the data from the first 2 days' bile collection differ from those collected over the subsequent 5 days. In the hypothyroid group, for instance, the average daily cholic acid excretion for the first 2 days was 13.1 mg, for the subsequent 5 days the average output was 27.0 mg; the average daily cholesterol excretion for this group for the first 2 days was 0.38 mg, and for the next 5 days was 1.0 mg.

Discussion. These data indicate that the amount of cholesterol excreted in the bile is a reflection of the level of thyroid activity, and as such, are in agreement with the findings of Rosenman, Friedman, and Byers(9). The absolute values for biliary cholesterol excretion in the present report, especially those in the hyperthyroid rat, are, however, considerably lower.

The excretion of cholic acid was found to be below control levels in both the hypo- and hyperthyroid animals, although a greater amount was excreted in the hypothyroid animals than in the hyperthyroid group. There was a gradual rise in the cholic acid output in the hypothyroid rats, reflecting perhaps the longer time needed by the hepatic excretory mechanisms of the propyl thiouracil-treated animals to return to normal. It is possible that the cholic acid excretion would return to control values in time.

It is of interest to note that following the first postoperative day, the cholic acid/cholesterol ratio for each of the 3 experimental groups remains quite constant and quite distinct.

Byers and coworkers have found that the cholesterol(14) and the cholic acid(15) output in the bile are uniformly higher on the first day following cannulation than during subsequent 24-hour periods. Our data, on the contrary, indicate that the biliary levels for these 2 substances rise following cannulation and reach a fairly constant plateau on the third or fourth postoperative day. It is evident from this, and from a study of the serial food intake, that the bile-cannulated animal does not approach a state of functional equilibrium until 3 or 4 days after cannulation.

Summary. 1. Hyper- and hypothyroid rats have been prepared and, along with euthyroid control rats, have been subjected to cannulation of the common bile duct and total daily bile collection for 7 days following cannulation. 2. Analysis of this bile for cholesterol and cholic acid revealed that the excretion of cholesterol is a reflection of the level of thyroid activity, and that cholic acid excretion is lowered in both hyper- and hypothyroidism. 3. After the third postoperative day, the ratio of cholic acid to cholesterol excreted in the bile was found to be relatively constant for each type of animal. 4. Cholic acid and cholesterol excretion in the bile was suppressed in both the hyper- and hypothyroid rats in the immediate postoperative period. In 3 or 4 days following cannulation, these levels reached a plateau.

---

1. Fisher, B., and Vars, H. M., *Am. J. Med. Sc.*, 1952, v222, 116.

2. Fisher, B., Zerbe, J., and Vars, H. M., *Surgical Forum, Clin. Congress Am. Coll. of Surg.*, W. B. Saunders Co., Philadelphia, 1952, p. 407.

3. Bloch, K., Berg, B. N., and Rittenberg, D., *J. Biol. Chem.*, 1943, v149, 511.

4. Byers, S. O., and Biggs, M. W., *Arch. Biochem. and Biophysics*, 1952, v39, 301.

5. Peters, J. P., and Van Slyke, D. D., *Quantitative Clinical Chemistry*, Williams & Wilkins Co., 2nd Edition v. 1, Baltimore 1946, p. 499.

6. Byers, S. O., Rosenman, R. H., Friedman, M., and Biggs, M. W., *J. Exp. Med.*, 1952, v96, 513.

7. Friedman, M., Byers, S. O., and Rosenman, R. H., *Circulation*, 1952, v5, 657.

8. Rosenman, R. H., Byers, S. O., and Friedman, M., *J. Clin. Endocr. and Metab.*, 1952, v12, 1287.

9. Rosenman, R. H., Friedman, M., and Byers, S. O., *Science*, 1951, v114, 210.

10. ———, *Circulation*, 1952, v5, 589.

11. Gurd, F. N., Vars, H. M., and Ravdin, I. S., *Am. J. Physiol.*, 1948, v152, 11.

12. Irvin, J. L., Johnston, C. G., and Kopala, J., *J. Biol. Chem.*, 1944, v153, 439.

13. Foldes, F. F., and Wilson, B. C., *Anal. Chem.*, 1950, v22, 1210.

14. Byers, S. O., and Friedman, M., *Am. J. Physiol.*, 1952, v168, 397.

15. Friedman, M., Byers, S. O., and Michaelis, F., *Am. J. Physiol.*, 1951, v164, 876.

Received May 13, 1953. P.S.E.B.M., 1953, v83.

Adsorption *in vitro* of Poliomyelitis Virus on Human Erythrocytes.* (20321)

C. W. Jungeblut,† B. Hofman, Jr., and J. D. Verlinde.

From the Department of Microbiology, College of Physicians and Surgeons, Columbia University, New York, and the Institute for Preventive Medicine, Leiden, Netherlands.

Among the neurotropic viruses adsorptive or hemagglutinating reactions have been described for lymphocytic choriomeningitis virus (1), Japanese B encephalitis virus(2), Theiler's mouse encephalomyelitis virus(3), and Col-SK virus(4). Little is known of the mechanism of interaction between these viruses and the red cell, except for Col-SK virus where the controlling factors have been analyzed in some detail(5-11). Classical poliomyelitis virus has given so far no evidence, through hemagglutination, of reacting *in vitro* with red cells. Yet viremia in poliomyelitis has been repeatedly demonstrated in experimentally infected animals(12-15) as well as in the human disease(16-18). Moreover, certain electronmicrographic alterations of the erythrocytes have been interpreted as indicating the presence of virus particles on the cellular surface(19,20). It became of interest, therefore, to investigate whether human poliomyelitis virus is capable of entering into specific interaction *in vitro* with red cells, as determined by viral adsorption.

Experimental. The virus chosen was the Leon strain of poliomyelitis virus.‡ Virus dilutions and cell suspensions were prepared with sterile potassium-veronal buffer(11). In preliminary experiments, 1 cc of virus dilutions of 1:100 was combined with 1 cc of 20% suspensions of washed human (O) or rabbit red cells and the mixtures were kept in the icebox (4°C) for several hours (4-16 hr). Following cold-centrifugation (15 minutes at 3000 R.P.M.) the red cells were separated from the supernatant fluids and the cellular sediments were resuspended in 2 cc of buffer solution or were laked by adding 2 cc of distilled water. Aliquots of the red cell preparations and of their corresponding supernatant fluids were tested for the presence of active virus by intracerebral injection into pairs of rhesus monkeys. Control animals received the test dose of virus which had been combined with buffer solution. The results of several experiments are summarized in Table I.

Results. These experiments show that active virus was uniformly present in the red cell sediment and in the supernatant fluid when human erythrocytes were used. When rabbit erythrocytes were used, virus was found on the red cells in one instance only but was regularly present in the supernatant fluid. Since the potency of Leon virus, as determined in control animals, reached ID_{50} values as high as 10^{-7}, it is evident that the red cells had contacted excessive amounts of virus in the original combination.

Another experiment was set up to study the quantitative aspects of the partition of virus between red cells and supernatant fluid in the original combination. Mixtures consisting of 2 cc of a 1:100 dilution of Leon virus and 2 cc of 20% suspensions of human or rabbit red cells were held in the icebox overnight and the virus content of the supernatant fluid was assayed by titration in monkeys. A control tube which contained 2 cc of a 1:100 dilution of virus in combination with 2 cc of buffer solution was included. The results are given in Table II.

It appears that the amount of free virus which could be detected in the supernatant decanted from the human red cell combination was at least 5 times less than that recovered from the supernatant decanted from the rabbit red cell combination. Since the latter equalled the titer found in the control, the human red cells had evidently removed an appreciable quantity of virus. No such re-

---

* Aided by grants from the Sister Elizabeth Kenny Foundation and the Dr. Simon Baruch Foundation.

† Fulbright scholar in the Netherlands, 1951-1952.

‡ We are indebted to Drs. H. A. Howe and D. M. Horstman for sending us samples of the Leon strain for experiments in Leiden and in New York.

TABLE I. Infectivity of Cells, before and after Washing, and of Their Corresponding Supernatant Fluids in Virus-Red Cell Combinations.

		Cell-virus combinations							
		Unwashed		1 × washed		2 × washed		3 × washed	
Red cells	Virus	Cells	S*	Cells	S	Cells	S	Cells	S
Human 20%	Leon 10^{-2}	7/8	7/7	1/4	0/2	1/4	0/2	9/10	0/7
Rabbit "	"	1/3	4/4	0/2	0/2			0/3	0/3

Virus controls: $10^{-2.3} = 7/7$; $10^{-3} = 3/4$; $10^{-4} = 6/6$; $10^{-7} = 2/4$; $10^{-7.33} = 0/3$; $10^{-8} = 0/2$

* S = Supernatant.
Denominator = No. of monkeys inj.; numerator = No. of monkeys paralysed.

TABLE II. Titration of Supernatant Fluid from Original Virus-Red Cell Combination.

Virus red cell combination		Titration of supernatant fluid		
Red cells	Virus	$10^{-2.3}$	10^{-3}	10^{-5}
Human 20%	Leon 10^{-2}	3/3	0/4	0/2
Rabbit "	"	3/3	2/2	0/2
Buffer control	"	3/3	2/2	0/2

Denominator = No. of monkeys inj.
Numerator = " " " paralysed.

action could be demonstrated with the rabbit red cells.

While the above results suggested that partial adsorption of virus had occurred on the human red cells, a possibility could not be dismissed that the cells had been contaminated by free virus in the ambient fluid. Further experiments were, therefore, carried out in which human or rabbit red cells, which had contacted virus, were washed repeatedly with large amounts of buffer solution. The washed cells and their corresponding supernatant fluids were then tested for the presence of active virus in the usual manner. Chilled reagents and glassware were used exclusively so as to minimize the chances of possible elution of the virus. Washing was accomplished by resuspending 0.2 cc of centrifuged, packed cells in 9.8 cc of buffer solution, agitating the suspension, and then recentrifuging it to obtain a new sediment. This procedure resulted in a 50-fold dilution of the contents in the original combination; by repeating the washing 3 successive times, a cumulative final dilution of 1:25,000,000 was obtained which was in excess of the verified endpoint of potency of the virus. The results obtained in these experiments are also recorded in Table I.

It will be seen that active virus was no longer demonstrable in any of the supernatant fluids decanted from either human or rabbit red cells after the first, second or third cycle of washing. On the other hand, the washed human cells were definitely infectious in several instances whereas similarly washed rabbit cells were non-infectious.

Summary and conclusions. When poliomyelitis virus was combined with human O erythrocytes at 4°C, active virus was present in the cellular sediment as well as in the supernatant fluid. Upon titration, the supernatant was found to contain less free virus than could be demonstrated in the supernatant from combinations of virus and rabbit erythrocytes, or in a buffer control. Human erythrocytes which, after contact with virus, had been washed repeatedly carried active virus whereas the corresponding supernatant was non-infectious. The described facts suggest that poliomyelitis virus may be adsorbed *in vitro* onto human erythrocytes.

---

1. Shwartzman, G., *J. Immunol.*, 1944, v48, 111.

2. Sabin, A. B., and Buescher, E. L., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 222.

3. Lahelle, O., and Horsfall, F. L., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 713; Lahelle, O., and Ward, T. J., *J. Immunol.*, 1951, v67, 75; Morris, M. C., *J. Immunol.*, 1952, v68, 97; 1953, v70, 37.

4. Hallauer, C., 4th Internat. Congr. Microbiology, July 1947, Copenhagen 1949, p. 257; Olitsky, P. K., and Yager, R. H., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 719.

5. Verlinde, J. D., and DeBaan, P., *Ann. Inst. Past.*, 1949, v77, 632.

6. Jungeblut, C. W., *Bull. N. Y. Acad. Med.*, 1950, v26, 571.

7. Jungeblut, C. W., Horvath, B., and Knox, A., *Arch. Ped.*, 1952, v69, 321.

8. Hallauer, C., *Arch. Virusforschg.*, 1951, v4, 224.

9. Bremer, A., *Ann. Inst. Past.*, 1951, v80, 40.

10. Vivell, O. and Mauer, R. Z., *Immun. Forschg.*,

1952, v109, 246.

11. Horvath, B., and Jungeblut, C. W., *J. Immunol.*, 1952, v68, 627.

12. Smith, M. G., Proc. Soc. Exp. Biol. and Med., 1943, v52, 88.

13. Verlinde, J. D., and Beem, B., *Ant. v. Leeuwenhoek*, 1952, v18, 1.

14. Horstman, D. M., Proc. Soc. Exp. Biol. and Med., 1952, v79, 417.

15. Bodian, D., *Am. J. Hyg.*, 1952, v55, 414.

16. Ward, R., Horstman, D. M., and Melnick, J. L., *J. Clin. Invest.*, 1946, v25, 284.

17. Koprowski, H., Norton, T. W., and McDermott, W., *U. S. Publ. Health Rep.*, 1947, v62, 1467.

18. Jungeblut, C. W., and Huenekens, E. J., to be published.

19. Mulé, F., *Istituto Superiore di Sanità, Roma,* 1952, v15, 220, 226, 230.

20. Reagan, R. L., Day, W. C., Harmon, M. P., and Brueckner, A. L., *Am. J. Path.*, 1953, v29, 175.

Received September 8, 1952. P.S.E.B.M., 1953, v83.

Increased Plasma 17-ketosteroid Concentration in Congenital Adrenal Hyperplasia. (20322)

Lytt I. Gardner. (With the technical assistance of Mrs. Sally Merrick.)

From the Department of Pediatrics, State University of New York Medical School, Syracuse.

Although there have been numerous studies of urinary 17-ketosteroid excretion in congenital adrenal hyperplasia(1-4), no data appear to be available on plasma concentration of 17-ketosteroids. Previous attempts to measure endogenous 17-ketosteroids in peripheral blood have resulted either in loss of the steroid during fractionation of plasma(5) or in overestimation of the material extracted(6). A method has recently been developed permitting estimation of endogenously produced 17-ketosteroids in peripheral plasma(7). Accordingly the plasma of normal adults and of patients with congenital adrenal hyperplasia has been investigated.

Methods. Oxalated or heparinized venous blood was collected. Ether extracts from acid-hydrolyzed whole plasma (10% HCl) were made and chromatographed on magnesia-silica gel columns, after which the residues were subjected to a micro-Zimmermann reaction(7). Optical density measurements were related to a calibration curve made with dehydroepiandrosterone acetate and the results expressed as μg 17-ketosteroids per 100 ml plasma. The spectrophotometric curves were done on the Zimmermann color of the residues prepared as described above, using a Beckman DU spectrophotometer (light path 1.0 cm, final vol. 0.5 ml). Recoveries of pure androsterone added to plasma samples carried through the extraction procedure varied between 85 and 100%. No 17-ketosteroids could be detected in the plasma of a gonadectomized-adrenalectomized adult. Prepubertal children were found to have plasma values of <3 μg %.

Results. Fig. 1 shows spectrophotometric curves of the Zimmermann reaction done on plasma steroid residues of a normal man age 35 years, and a girl with untreated congenital adrenal hyperplasia age 13 years. The maxima at approximately 520 mμ are noteworthy,

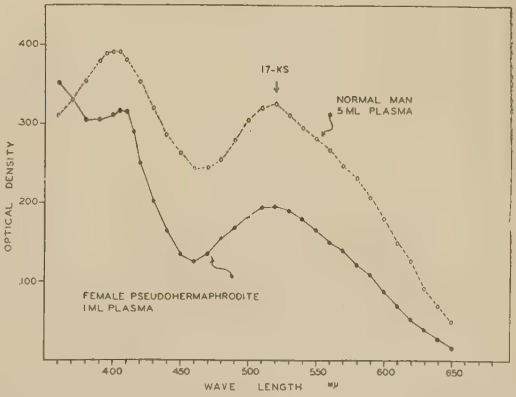


FIG. 1. Spectrophotometric curves of the Zimmermann reaction on plasma residues from a man age 35 years and a girl with congenital adrenal hyperplasia age 13 years (case Gr).

TABLE I. Plasma and Urine 17-Ketosteroid Values.

Patient	Sex	Age, yr	Diagnosis	Neutral 17-ketosteroids Plasma, μg/100 ml	Urine, mg/24 hr
Gr.	♀	13	Congenital adrenal hyperplasia	360	300
Ci.	♂	7	" " "	80	29
An.	♂	8	" " "	80	49
Gu.	♀	6	" " "	88	42
15 adult ♂ controls		19-35	Normal	74 ± 7*	9-17

* Mean ± stand. error of the mean.

since 17-ketosteroids absorb maximally in this spectral range. The significance of the maxima at 405 mμ is not known at present.

Table I presents quantitative data on the concentrations of plasma 17-ketosteroids and urinary 17-ketosteroid excretion in 4 children with untreated congenital adrenal hyperplasia as compared with 15 normal men. It will be noted that patient Gr., a female pseudohermaphrodite, has 17-ketosteroid concentrations in plasma and urine very greatly in excess of the values shown by the younger boys with congenital adrenal hyperplasia. The reason for this difference is not known, although it has previously been observed that the urinary excretion of 17-ketosteroids in this disease may show a marked increase at the time of epiphyseal closure(8). It is apparent that the plasma as well as urine 17-ketosteroid concentrations of these children are considerably in excess of the mean value for normal adult males.

When patient Ci was given 50 mg of cortisone per day by mouth, his plasma 17-ketosteroid concentration fell to values of <16 μg %, and urine values to <8 mg per day.

Discussion. The observation that peripheral plasma 17-ketosteroids are elevated in untreated cases of congenital adrenal hyperplasia and fall with cortisone therapy is of interest in view of what is presently known about plasma 17-hydroxycorticosteroid concentrations. Nelson(9) and Kelley *et al.*(10) have noted that plasma 17-hydroxycorticosteroid concentration is abnormally low in untreated cases of congenital adrenal hyperplasia, and rises to normal values when cortisone therapy is begun. Thus, there appears to be a reciprocal movement of plasma concentrations of 17-ketosteroids vs. 17-hydroxycorticosteroids when patients with this disease are treated with cortisone.

Summary. Measurements of peripheral plasma 17-ketosteroids were made in 15 normal men and in 4 children with untreated congenital adrenal hyperplasia. The 4 children showed plasma values equal to or in excess of the values shown by the men. When one of the children was treated with cortisone, plasma 17-ketosteroid concentration fell to values far below the adult level.

The author is indebted to Dr. Charles Lloyd in whose laboratory the urinary 17-ketosteroid determinations were done and who made possible the observations on patient An. Dr. Elmer Alpert of Merck and Co. kindly provided the Cortone used in this study. Pure dehydroepiandrosterone acetate was generously furnished by the Schering Corp.

---

1. Talbot, N. B., Butler, A. M., and MacLachlan, E. A., *New England J. Med.*, 1940, v223, 369.
2. Wilkins, L., Lewis, R. A., Klein, R., Gardner, L. I., Crigler, J. F., Jr., Rosemberg, E., and Migeon, C. J., *J. Clin. Endocrinol.*, 1951, v11, 1.
3. Bartter, F. C., Forbes, A. P., and Leaf, A., *J. Clin. Invest.*, 1951, v30, 237.
4. Gardner, L. I., and Migeon, C. J., *J. Clin. Endocrinol. and Metab.*, 1952, v12, 1117.
5. West, C. D., Tyler, F. H., Brown, H., and Samuels, L. T., *J. Clin. Endocrinol.*, 1951, v11, 897.
6. Dumazert, C., and Valensi, G., *Comptes Rendus Soc. de Biol.*, 1952, v146, 471; also cf. Zimmermann, W., *Vitamine u. Hormone*, 1944, v5, 276.
7. Gardner, L. I., *J. Clin. Endocrinol. and Metab.*, 1953, v13, n8 (Aug.).
8. Gardner, L. I., Sniffen, R. C., Zygmuntowicz, A. S., and Talbot, N. B., *Pediatrics*, 1950, v5, 808.
9. Nelson, D. H., personal communication.
10. Kelley, V. C., Ely, R. S., and Raile, R. B., *J. Clin. Endocrinol. and Metab.*, 1952, v12, 1140.

Received April 6, 1953. P.S.E.B.M., 1953, v83.

Rate of Excretion of Glutamyl Polypeptide and Its Polymers in Humans.
(20323)

Max Bovarnick, Stanley Fieber, Marianna R. Bovarnick, and Joseph Kazlowski. (Introduced by P. H. Long.)

From the Veterans Administration Hospital, Brooklyn, N. Y. and The Department of Medicine, College of Medicine, State University of New York, New York City

Further work on the polyglutamic acid previously investigated by us with reference to its mode of formation(1) has revealed characteristics relating to the use of this peptide and its derivatives as a plasma volume extender. The one major defect is its rapid disappearance from the blood stream and high rate of excretion by the kidney. Thus, the glutamyl peptide, prepared as previously described(1) with a molecular weight of 12,000 to 15,000, is completely cleared from the blood stream of humans within 2 hours after injection. As much as 85% of the injected material is found in the urine within 3 or 4 hours after injection.

Since rate of excretion from the blood stream is a function of such molecular properties as size, shape, surface configuration and charge, various derivatives of the peptide have been prepared and studied to provide a correlation of physiological properties with such physico-chemical properties. One result obtained of considerable interest in the problem of plasma volume extenders is concerned with molecular diameter and rate of renal excretion of these macromolecules. This result, as demonstrated in humans, is reported herewith.

Materials. The substances tested were glutamyl polypeptide and glutamyl polypeptide polymerized with some of its partial degradation products. The molecular dimensions of the glutamyl polypeptide were estimated to be 11 A by 150-200 A(1). The average number molecular weight of the glutamyl peptide was 12000-15000, that of the partial degradation products was 2000-3000. These molecular weights were obtained by both formol titration of the terminal amino group and the dinitrophonyl fluoride end group determination of Sanger(3), the methods agreeing within 10%. The molecular weights of the polymers have not yet been accurately determined, but are estimated to be 80000 on the basis of data obtained during their preparation. The method of preparation of these polymers, as well as a method for the quantitative determination of both the peptide and these polymers, will be published elsewhere(2). There was no notable change in molecular length, the increase in molecular weight being associated with increase in molecular diameter. Both *glutamyl peptide and its polymers* were injected intravenously as 3% solutions of sodium salt in isotonic saline. Sterilization was achieved by filtration. All solutions were tested for pyrogenicity and sterility prior to use. Each subject was administered 3 g of the material being tested during the course of one hour. Control analyses for blood and urine level were taken prior to injection. Blood levels were taken 10 minutes after the end of injection, and then at suitable intervals. Urinary excretion of the material was determined from the start of the injection and followed at least 24 hours.

Results. It is evident in Fig. 1 that the

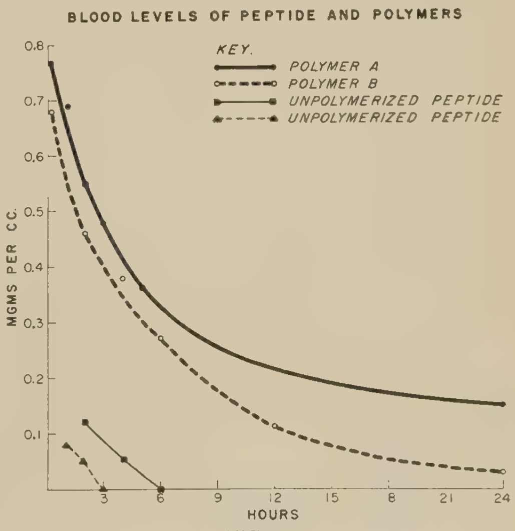


FIG. 1.

---

* We are indebted to Merck and Co. for a gift of the glutamyl peptide used in this work.

half-life of the glutamyl peptide in the human blood stream is increased by polymerization. As a corollary, the rate at which the polymerized material is excreted in the urine is decreased. It is pertinent to state here that there was no significant change in temperature, pulse, or respiration in either subject during the course of administration or at any time during the next two days. Subjectively the patients experienced no symptoms whatsoever.

It is evident that the rate of disappearance of the polymer of glutamic acid from the human blood stream can be decreased by increasing the diameter of the molecule, other factors such as the length and composition of the molecule being kept constant. The molecular length of both the peptide and the polymer are approximately that of serum albumen.

Summary. The half-life of glutamyl polypeptide in the blood stream of humans can be increased by polymerization leading to an increase in the molecular diameter.

---

1. Bovarnick, M., *J.B.C.*, 1942, v1451, 415.

2. Fieber, Stanley, Bovarnick, Marianna R., Kazlowski, Joseph, in press.

3. Sanger, F., *Biochem. J.*, 1951, v49, 23.

Received April 7, 1953. P.S.E.B.M., 1953, v83.

Butazolidin Plasma Concentration and Urinary Excretion in Normal Individuals and in Patients with Rheumatoid Spondylitis.* (20324)

ALICE BORDEN STANFIELD, EMILY C. BRODIE, AND ELMER E. YEOMAN.
(Introduced by A. R. Kemmerer.)

From the Department of Nutrition, University of Arizona, and the Southwestern Clinic and Research Institute, Tucson.

The current use of 3,5-dioxo-1,2-diphenyl-4-n-butyl pyrazolidine (Butazolidin)† in the treatment of rheumatic diseases has become widespread in this country as well as in Europe. Although there are many references regarding its use and the resultant clinical response(1-4) there has been no satisfactory report concerning plasma concentration and the urinary excretion of the drug. At the time of the initial report on the use of Butazolidin in 188 patients(3), information regarding the absorption of the drug was not available. It was known that the absorption was occasionally incomplete because some patients observed the intact tablets in the stool. Studies on drug concentrations were instituted in an attempt to establish definite absorption, optimum dosage, and to arrive at a possible explanation of the toxic reactions frequently encountered during therapy(2,4-6). Experience indicates that Butazolidin is more effective in the treatment of rheumatoid spondylitis than in any other type of rheumatic disease. For that reason this study was limited to the results from treated spondylitis patients.

This paper reports plasma concentrations and urinary excretion of Butazolidin for 11 normal individuals and 6 patients with rheumatoid spondylitis who were treated in the same manner. Rates of absorption and disappearance of the drug in plasma and urine are reported for one patient with osteo arthritis.

Methods. The normal control group consisted of 6 male and 5 female university students between the ages of 19 and 25. Each individual was carefully instructed and later checked to make certain that his diet was of adequate caloric and vit. C content as well as one which supplied a minimum of one gram of protein per kilogram of body weight. All meals were provided at the same dining hall on the campus. Twenty-four hour urine samples were collected on 2 consecutive days and

---

* This work was supported in part by grants from the U. S. Public Health Service and the Fair Foundation, Arizona Agric. Exp. Station Technical Paper No. 325.

† Butazolidin was supplied by Geigy Pharmaceuticals, Geigy Co., New York.

a blood specimen on one day preceding institution of therapy. A routine urinalysis, a routine blood count, platelet count and erythrocyte sedimentation rate were performed at the beginning of the experiment. After a 2-day control period on the diet, 400 mg of Butazolidin were administered orally in 2 divided daily doses. The students were instructed that medication must always be taken following meals and that any unusual symptoms be reported at once to the physician in charge. During the treatment period of 10 days, blood specimens and 24-hour urine samples for Butazolidin levels were obtained on the 1st, 3d, 5th, 7th, and 10th days. Approximately 12 hours elapsed between time the last medication was ingested and the blood sample obtained. Five male and 1 female patients with indisputable rheumatoid spondylitis were similarly treated. The patients ranged in age from 19 to 40 years. The patient with osteo arthritis had been on a daily dose of 400 mg of Butazolidin for 2 weeks. At the time the medication was discontinued the plasma level was 10.3 mg % and the rate of disappearance of the drug from both plasma and the urine was carefully followed for 12 days. On the 15th day the drug was started again and its reappearance and rise in the urine and plasma was studied for an additional 18 days. On the day the specimens were obtained, determinations for each individual were estimated in duplicate. In the plasma procedure the original method was modified in order to eliminate the formation of permanent emulsions. Ground glass stoppered test tubes of 50.0 ml capacity were substituted for glass bottles and antifoam‡ was added to the mixture before shaking. The final estimations were made by reading the optical density at 265/u in a Model DU Beckman Spectrophotometer. Recoveries of Butazolidin added to plasma and to urine were satisfactory (100% ± 4%).

Results. Butazolidin was demonstrated in both plasma and urine in all 17 subjects. Its definite absorption from the intestinal tract was thus established. With the exception of female students W. O. and S. A. no other individuals gave evidence of any toxic reactions to the drug. No member of the group of normal students reported loss of appetite, symptoms of gastric disturbance, insomnia or euphoria. There was no development of edema and no significant weight gain. No symptoms were detected which could be referred to ingestion of Butazolidin. The results for normal stu-

TABLE I. Butazolidin Plasma Concentrations.*
6 patients with rheumatoid spondylitis.
Control = 0 mg %.

Subjects	Days on treatment 1	3	5	7	10
	mg %				
K. K.	3.8	9.3	9.6	14.0	13.4
S. G.	.8	4.1	11.3	13.2	12.6
R. N.	1.0	4.6	7.4	9.1	10.2
B. P.	1.6	7.4	9.8	8.2	11.9
N. D.	1.2	7.7	12.8	13.7	13.1
Mc. A.	.0	10.7	11.4	11.8	12.4
Avg	1.4	7.3	10.5	11.7	12.3
	11 normal students				
B. B.	3.8	6.2	11.0	12.6	14.0
D. D.	3.3	3.8	8.8	16.1	15.3
P. N.	3.9	7.2	8.9	11.9	14.5
S. L.	2.6	5.6	9.5	11.2	14.9
W. R.	3.5	7.6	12.4	14.4	12.8
W. L.	.0	5.8	11.5	12.7	13.8
B. W.	4.7	11.7	14.4	14.5	15.8
Mc. D.	2.6	8.2	9.5	11.7	14.5
S. D.	.0	6.8	7.7	14.7	13.1
Avg	2.7	7.0	10.4	13.3	14.3

* Daily dose 400 mg Butazolidin.

TABLE II. Butazolidin Urinary Excretion Values.*
6 patients with rheumatoid spondylitis.
Control mg/24 hr = 0.

Subjects	Days on treatment 1	3	5	7	10
	mg/24 hr				
K. K.	2.5	2.8	27.1	21.2	37.2
S. G.	.0	2.8	9.6	35.6	34.0
R. N.	.0	4.3	10.0	13.7	15.3
B. P.	.0	2.2	9.8	16.1	17.6
N. D.	.0	1.4	15.2	32.4	25.9
Mc. A.	.0	5.1	5.1	19.7	36.9
Avg	.4	3.1	12.8	23.1	27.8
	11 normal students				
B. B.	.0	3.2	20.3	32.3	41.4
D. D.	.0	3.8	16.9	20.4	27.8
P. N.	.0	.0	2.2	7.4	23.6
S. L.	.0	2.2	11.1	17.2	14.3
W. R.	.0	.6	10.5	12.4	43.8
W. L.	.0	2.2	13.0	19.2	30.3
B. W.	2.0	7.0	18.8	46.8	69.0
Mc. D.	.0	2.9	7.7	17.7	48.1
S. D.	.0	2.2	9.0	21.0	17.3
Avg	.2	2.7	12.2	21.6	35.1

* Daily dose 400 mg Butazolidin.

---

‡ Dow Corning Antifoam A. Silicone Defoamer.

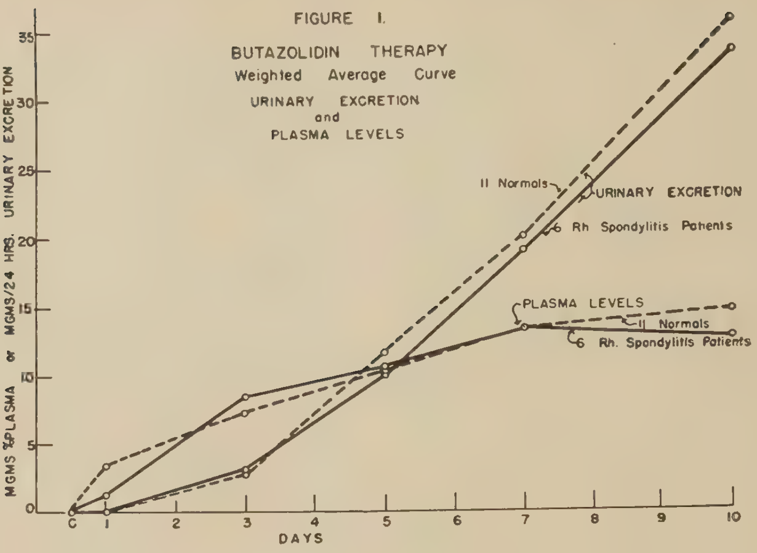


FIGURE I.
BUTAZOLIDIN THERAPY
Weighted Average Curve
URINARY EXCRETION
and
PLASMA LEVELS

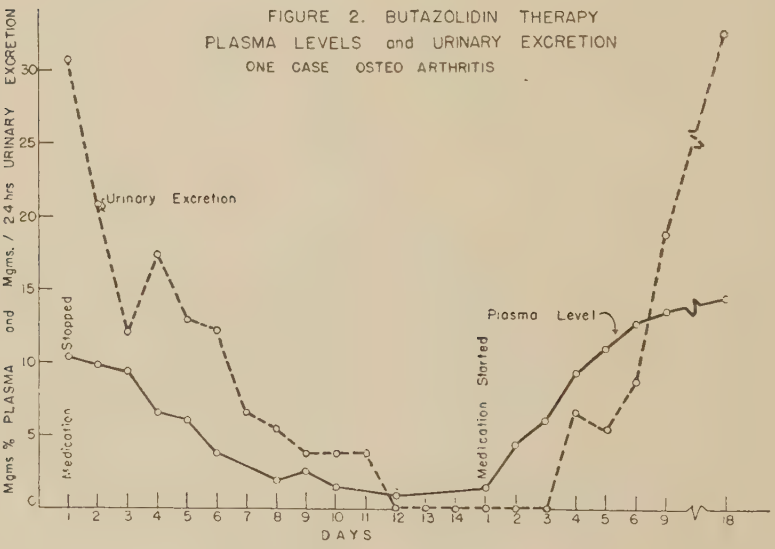


FIGURE 2. BUTAZOLIDIN THERAPY
PLASMA LEVELS and URINARY EXCRETION
ONE CASE OSTEO ARTHRITIS

dents, W. O. and S. A. were eliminated from all but the weighted mean calculations. Butazolidin concentrations in the plasma for the remaining 9 normal students and for the group of patients with rheumatoid spondylitis are presented in Table I.

Butazolidin urinary excretion values for the two groups studied appear in Table II.

Fig. 1 represents the weighted average graph§ of both plasma concentration and urinary excretion values for the 2 groups. On the 10th day the difference in plasma levels in the 2 groups was significant at the 1% level. At no other time were the differences significant in either plasma concentration or urinary excretion values. The average weight of the group of normal individuals was 22 pounds higher than the average weight of the patients with rheumatoid spondylitis. When individual values for all subjects were arbitrarily converted to gamma per pound of body weight instead of mg % the significant value mentioned above was no longer evident. We believe it can be explained on a weight difference basis and should be disregarded. The conversion to gamma per pound of body weight did not alter the fact that none of the differences in urinary excretion values was significant.

---

§ The weighted mean derived by averaging remaining values after discarding those which fell outside the mean plus or minus the standard deviation. (Values between $X \pm S_x$).

On the 10th day of treatment the weighted mean average Butazolidin plasma level for both groups was 14.0 mg % or roughly 350 mg in the total circulating plasma. This represents approximately 88% of a daily dose. On the same day the weighted average urinary excretion value for the two groups was 35.0 mg/24-hours or approximately 8.8% of a daily dose. Fig. 2 shows a curve representing the daily rates of disappearance of Butazolidin in the plasma and urinary excretion for 1 patient with osteo arthritis. Butazolidin did not disappear from the urine until the 12th day after the drug was discontinued. A total of 129 mg had then been excreted. On the 3d day after Butazolidin had completely disappeared from the urine, medication was started again in a daily dose of 400 mg. It did not reappear in the urine until the 4th day following reinstitution of therapy.

Although the presence of Butazolidin was not detected in the urine after 12 days it was present in the plasma in small amounts (0.84-1.24 mg %) even 15 days after the drug was discontinued.

Discussion. This study was not designed to show differences in response to Butazolidin therapy between normal individuals and patients with rheumatoid spondylitis. Rather, both groups were incorporated for the purpose of establishing information on the absorption and urinary excretion of the drug. The results show that Butazolidin was absorbed from the intestinal tract in all individuals treated. Not until the 7th and 10th days of treatment was the plasma level in any way commensurate with the daily drug intake. When the urinary excretion value was highest only about 10% of the daily dose of butazolidin was being excreted as such.

It is interesting to note the slow disappearance of the drug from both the plasma and the urine in the patient with osteo arthritis. On the day the medication was discontinued there were roughly 300 mg in the total circulating plasma. In the 12 days required for the Butazolidin to disappear completely from the urine, only 129 mg were excreted. The drug was still present in small amounts in the plasma on the 15th day when medication was again started.

A number of possibilities have been considered to explain the disposition of Butazolidin by the body but as yet we have little actual proof to substantiate them.

Summary. 1. Four hundred mg of Butazolidin were administered orally for 10 days to 11 normal students and to 6 patients with rheumatoid spondylitis. Plasma concentrations and total 24-hour urinary excretion values for the drug were estimated. 2. Butazolidin was demonstrated in the plasma of all individuals between the 1st and 3d days on treatment and appeared in the urine between the 1st and 5th days thus proving its definite absorption in all 17 subjects. With the exception of 2 mild cases of edema in 2 females in the normal group, no toxic effects of the drug were encountered during therapy. There were no significant differences in the reactions in the 2 groups. No sex differences were noted in the rate of absorption or excretion of the drug although the number of subjects admittedly was small. 3. Butazolidin did not completely disappear from the urine in one patient with osteo arthritis until 12 days following cessation of therapy and it was still detected in small amounts in the plasma at the end of 15 days. 4. We have at this time no adequate explanation regarding the disposition of the drug by the body although there is some evidence to suggest that a part of it is stored as Butazolidin in the tissues or carried in the circulating blood.

The authors wish to express their appreciation to Dr. Bernard B. Brodie and to Dr. John J. Burns of New York University College of Medicine for their preliminary method for the determination of Butazolidin in plasma and urine. Further appreciation is extended to the group of students for their excellent cooperation in this study and to Mr. Joel E. Fletcher for his help with the statistical analyses and charts. The technical assistance of Mrs. Marjorie Denney, Mrs. Verona McKibbin and Mrs. Lessie Kirch is gratefully acknowledged.

1. Kuzell, W. C., Schaffarzick, R. W., Brown, B., and Mankle, E. Z., *J. Am. Med. Assn.*, 1952, v149, 729.

2. Steinbrocker, Otto, Berkowitz, Sidney, Erlich, Mortimer, Elkind, Mortimer, and Carp, Solomon, *J. Am. Med. Assn.*, 1952, v150, 1087.

3. Stephens, Charles A. L., Jr., Yeoman, Elmer E., Holbrook, W. Paul, Hill, Donald F., and Goodwin, William L., *J. Am. Med. Assn.*, 1952, v140, 1084.

4. Hart, Dudley F., and Johnson, Alan M., *The Lancet*, 1952, v2, 43.

5. Hinz, Carl, Lamont-Havers, Ronald W., Cominsky, Burton, and Gaines, Leonard M., *J. Am. Med. Assn.*, 1953, v151, 38.

6. Loston, Geoffrey E., Le Vay, David, Wilson, Peter, *The Lancet*, 1952, v2, 682.

Received April 21, 1953. P.S.E.B.M., 1953, v83.

Effect of Initial Length on the Work of the Cardiac Atrium.* (20325)

ROBERT C. LITTLE, S. FRED STRAIN, JR., AND ROY D. SCHAEFER.†
From the Division of Physiology, University of Tennessee, Memphis.

Atrial contraction may insure adequate cardiac filling in patients with mitral or tricuspid stenosis. Factors that affect the work done by atrial systole became of great importance in such cases. The relationship between ventricular initial length and systolic work has been studied(1-2); apparently similar relationships for mammalian atrial muscle have not been investigated. Such information may be valuable in interpreting atrial pressures, particularly in cases of valvular dysfunction.

Method. Strips of atrial muscle 2 by 0.5 cm were removed carefully from the beating left atrium of dogs anesthetized with sodium barbital. The strips were placed in warm Tyrode's solution modified to contain 0.008% NaH_2PO_4 and 0.013% $CaCl_2$. The solution was oxygenated with 95% O_2 and 5% CO_2, and maintained at 39°C. One end of the muscle strip was fixed by a support and the other end suspended by a simple lever. Weights ranging from 0.5 to 10 g were placed on the free arm of the lever, and the resulting muscular stretch was recorded by projecting the movement of the lever onto a photokymograph. Muscular contractions were elicited by maximal induction shocks, and the height of the contractions was recorded. All records were corrected for magnification and were expressed as the actual movement in mm of the free end of the muscle strip. The work performed by each contraction was calculated in gram-centimeters. Muscular stretch was calculated as percent of the original 2 cm length.

Results. A total of 131 observations on strips from 9 atria were satisfactory for analysis. The amount of work performed varied widely among muscular strips. However, in each case the amount of work increased with increase in initial length until a

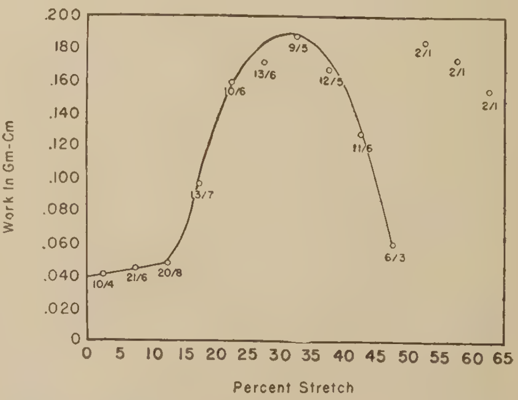


FIG. 1. Work-stretch diagram of left atrial strips. Circles are avg work performed. Upper figures are number of observations, lower figures are number of animals. The three points in upper left of plot were all from the same animal.

---

* Supported by a Research Grant from the National Heart Institute, National Institutes of Health, U. S. Public Health Service.

† Postdoctorate Research Fellow, U. S. Public Health Service.

maximum work response was obtained.

The degree of stretch was divided into groups; the class interval being a 5% increase. Plotting average amount of work done by the muscle for each group (Fig. 1) indicates the maximum work response occurs at 30% stretch over the original length. This agrees closely with the mean of the maximum for each individual muscle strip, the mean being 26.9% with a standard deviation of 9.7.

Discussion. The average filling volume of the left atrium of the dog is stated(3) as 3.4 cc. Assuming that the atria are spherical, this gives a surface of 10.93 square cm. To increase this surface 30%, it is necessary to increase the volume to 5.03 cc. Calculations employing previously reported(3) volume elasticity curves of the left atrium indicate that this volume will result in an intra-atrial pressure of 150 mm of H_2O.

Such a theoretical analysis of the data indicate that maximum left atrial systolic work will occur at an atrial pressure of 150 mm of H_2O. Pressures higher than this will result in atrial failure with markedly reduced work output.

Summary. Strips of left atrial muscle from the dog heart were subjected to varying degrees of stretch, and the work performed during contraction was calculated. Maximum work occurred at approximately 30% stretch. A theoretical analysis of these data indicates that maximum left atrial systolic work occurs at an intra-atrial pressure of 150 mm of water.

1. Patterson, S. W., Piper, H., and Starling, E. H., *J. Physiol.*, 1914, v48, 465.
2. Starling, E. H., and Visscher, M. D., *J. Physiol.*, 1927, v62, 243.
3. Little, R. C., *Am. J. Physiol.*, 1949, v158, 237.

Received April 23, 1953. P.S.E.B.M., 1953, v83.

Effect of Pituitary Extracts on Radiation-Induced Testicular Atrophy in Rats.* (20326)

JOHN B. STORER AND PHYLLIS SANDERS. (Introduced by J. M. Coon.)
From the University of California, Los Alamos Scientific Laboratory, Los Alamos, N. M.

Treatment of both local and total-body radiation injuries in man and experimental animals has, in general, produced disappointing results. One exception to such results has been the study of Momigliano and Essenberg (1) on the effect of pituitary extract on radiation-induced testicular atrophy in rats. They have reported that gonadophysin (Searle), an extract from sheep pituitaries which contains both follicle-stimulating and luteinizing hormone, hastens regeneration of the testes of albino rats when injections are begun shortly after radiation exposure and continued for several weeks.

This finding is at variance with the usual experiences in treating radiation injuries and because of the possible practical importance of this method of treatment in human cases it was considered worth while to repeat and extend this work.

Materials and methods. A total of 172 three-month-old and 48 thirty-day-old male Sprague-Dawley rats weighing 280 to 340 and 80 to 110 g, respectively, were used. The animals were maintained 5 or 6 to a cage and given water and Purina laboratory chow *ad libitum*. The details of the method of X-ray exposure have been given in a previous report(2). In the experiments in which body shielding was employed the animals were anesthetized with pentobarbital (40 mg/kg), their testes drawn into the scrotum, and their entire bodies, with the exception of the scrotum, shielded with 6.4 mm of lead during the X-ray exposures. Gonadophysin (Searle), containing both follicle-stimulating hormone (FSH) and luteinizing hormone (LH) which are stimulatory to the seminiferous tubules and interstitial cells of the testis, respectively, was injected subcutaneously twice a week.

---

* Work done under the auspices of the AEC.

TABLE I. Effect of Gonadotropins on X-Ray-Induced Testicular Atrophy in Adult Rats Exposed to 500 r Total Body X Radiation. 6 rats in each series.

Treatment	Time of sacrifice, wk after x-radiation	Body wt, g	Wt of testes, g/100 g body wt	Testes, % of control wt
None (controls)	4	366	.97	100
	6	372	.95	100
	8	379	.97	100
Unirradiated 12.5 RU gonadophysin biweekly	4	369	.96	99
	6	393	.91	96
	8	400	.80	82
500 r total-body X radiation	4	361	.62	64
	6	362	.39	41
	8	382	.42	43
500 r total-body X radiation plus 12.5 RU gonadophysin biweekly	4	352	.58	60
	6	354	.36	38
	8	376	.31	31

TABLE II. Effect of Gonadotropins on X-Ray-Induced Testicular Atrophy on Adult Rats Exposed to 400 r Total-Body X Radiation. 5 rats in each series.

Treatment	Time of sacrifice, wk after x-radiation	Body wt, g	Wt of testes, g/100 g body wt	Testes, % of control wt
None (controls)	4	329	1.09	100
	6	345	1.10	100
	8	401	.92	100
	12	388	.94	100
	16	391	.92	100
Unirradiated 12.5 RU gonadophysin biweekly for 4 wk	4	308	1.08	99
	6	340	.91	93
	8	384	.87	95
	12	412	.86	91
	16	377	.88	96
500 r total-body X radiation	4	267	.81	74
	6	318	.45	41
	8	310	.40	43
	12	357	.38	40
	16	327	.47	51
500 r total-body X radiation plus 12.5 RU gonadophysin biweekly for 4 wk	4	294	.61	56
	6	314	.37	34
	8	353	.34	37
	12	329	.40	43
	16	328	.54	59

At predetermined time intervals rats were killed with ether and their testes, ventral prostate, and seminal vesicles removed and weighed. Two groups of adult male rats were given a single total-body exposure to 500 r of X-rays. Immediately after exposure and biweekly thereafter the animals were given 12.5 RU (rat units) of gonadophysin subcutaneously. In one group the injections were continued to the time of sacrifice at 4, 6, and 8 weeks after radiation exposure. The second group received the injections for 4 weeks. Rats from this group were sacrificed at 4, 6, 8, 12, and 16 weeks. Two groups of immature rats were given 800 r in 3 equally divided doses 5 days apart, with the first dose given when the rats were 30 days old. In each exposure the entire body with the exception of the scrotum was shielded with lead. Immediately following the last exposure one of the groups was given biweekly injections of 10 RU of gonadophysin for 8 weeks. At the end of this time interval the rats were sacrificed. The second group of immature rats was given biweekly injections of 10 RU of gonadophysin beginning 8 weeks following the last X-ray

TABLE III. Effect of Gonadotropins on Testicular Atrophy and Regeneration following 800 r (Fractionated in 3 Equal Doses) to the Scrotum of Immature Rats. 6 rats in each series.

Treatment	Time of sacrifice, wk after last dose of x-radiation	Body wt, g	Wt of testes, g/100 g body wt	Testes, % of control wt
None (controls)	8	372	.95	100
Unirradiated 10 RU gonadophysin biweekly from time of X radiation to sacrifice	8	339	.91	96
800 r to scrotum in 3 doses beginning when rats were 30 days old	8	318	.41	43
Radiation as above. 10 RU gonadophysin biweekly from time of last X radiation to sacrifice	8	320	.36	38
None (controls)	16	380	.93	100
Unirradiated 10 RU gonadophysin eighth to 16th wk	16	354	.83	90
800 r to scrotum in 3 doses beginning when rats were 30 days old	16	339	.81	87
Radiation as above. 10 RU gonadophysin biweekly 8th to 16th wk following last dose of X radiation	16	347	.35	38

exposure and continuing for an additional 8 weeks. This latter group was included to test the effect of the hormones on regenerating testes. In all the above studies 3 control groups of rats were used. These included normal rats of the same age as the experimental animals, rats receiving a similar dose of X-rays but no injections, and rats receiving injections of gonadophysin but no X-ray exposure.

Results. No evidence was obtained in the present study that gonadophysin injections exerted any beneficial effect on testicular atrophy or regeneration in rats following X-radiation. The data are summarized in Tables I-III. In general the degree of atrophy appeared to be somewhat greater in the irradiated rats receiving gonadophysin than in those receiving only X-radiation. This finding seemed to hold for both the immature rats receiving fractionated radiation to the testes alone and for adult rats receiving a single total-body exposure. Regeneration of the testes was incomplete by 16 weeks following irradiation in the case of adult rats exposed to 500 r, although the data shown in Table II suggest that regeneration was progressing both in the animals exposed only to X-rays and in those exposed to X-rays and injected with gonadophysin. The small differences in degree of regeneration are not considered significant in view of the individual variations found between rats of the same group.

In the case of 30-day-old rats exposed to 800 r in fractionated doses, regeneration was practically complete by 16 weeks following irradiation. It is apparent from Table III that when gonadophysin was administered during the time when regeneration was occurring in the irradiated controls (8th to 16th week), such regeneration was completely inhibited in the gonadophysin-treated rats.

The weights of the accessory sex organs are not included in the tables since in no instance was there a significant difference between the weights found for gonadophysin-injected and uninjected rats. The changes in weight of these organs following X-radiation paralleled the changes seen in the testicular weights.

Gonadophysin alone was found to produce a slight but consistent degree of testicular atrophy in all the groups studied (Tables I-III).

Discussion. Injections of gonadophysin neither prevented atrophy nor increased regeneration in the testes of adult rats exposed to 500 r of X-radiation or of immature rats exposed to 800 r to the testes in divided doses. With each dosage schedule the gonadophysin slightly intensified the atrophy. Regeneration of the testis was inhibited in the experiment in which gonadophysin was given during the period in which regeneration would normally occur. This observation is in disagreement with that of Momigliano and Essenberg, who found a beneficial effect from injection of these hormones(1). In that study, 30-day-old rats were given 800 r to the scrotum in 3 divided doses at the same intervals employed in the present experiments. The injections given were similar although the time intervals used by Momigliano and Essenberg differed slightly from those used here. From their description of the changes induced it is evident that far more degeneration was seen by them than was found in the present study. The reasons for these discrepancies are not apparent.

Five hundred roentgens delivered in single doses to the entire body of adult rats produced more severe testicular atrophy and slower regeneration than did 800 r in 3 divided doses delivered only to the testes of immature rats. It is not known as yet whether this effect is due to additional deleterious effects produced by total body irradiation, such as disturbance of other hormone systems, anemia, weight loss, etc., whether fractionating the dose simply produces less effect even though the total amount given is higher, or whether the age of the animals is responsible for the difference.

Neither dose of X-radiation was sufficient to produce sterilization. Microscopic sections which were taken in all cases indicated that some mature spermatozoa were present. It is not known whether gonadotropic hormones would exert any beneficial effect on rats given enough X-radiation to produce complete sterility. Such an effect seems doubtful in view of the fact that at lower doses atrophy is intensified by the injections.

The interstitial cells of the testes are generally believed to be relatively resistant to radiation damage(3-7). A number of investigators have prevented atrophy of the accessory sex organs following irradiation by injecting compounds rich in leuteinizing hormone(8-10). There have also been reports of questionable hyperplasia of interstitial cells following irradiation alone(4). In the present study there was no histologic evidence of radiation damage to the interstitial cells. It appeared, however, that there was a functional depression since the accessory organs which depend on androgens secreted by the interstitial cells for their maintenance of normal size become atrophic. Injections of gonadophysin, a compound containing luteinizing hormone, did not prevent atrophy of these accessory organs in X-rayed rats even though luteinizing hormone is believed to stimulate specifically the interstitial cells and increase the androgen output.

Summary. A study has been made on the effect of gonadophysin (Searle), an extract from sheep pituitaries which contains both follicle-stimulating and luteinizing hormone, on radiation-induced testicular atrophy in rats. Adult and immature male rats were given single exposures of 500 r total-body X-radiation and 800 r of X-radiation to the testes only in 3 fractionated doses, respectively. Gonadophysin was injected twice weekly for the first 4 to 6 weeks or from the 8th to 16th week following radiation exposures to evaluate the effectiveness of this agent on testicular atrophy and regeneration. Gonadophysin was found to increase slightly the degree of testicular atrophy and to inhibit testicular regeneration in rats.

---

1. Momigliano, E., and Essenberg, J. M., *Radio.,* 1944, v42, 273.

2. Storer, J. B., Sanders, P., and Lushbaugh, C. C., PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 541.

3. Heller, M., In *Histopathology of Irradiation from External and Internal Sources,* ed. by W. Bloom, McGraw-Hill, New York, 1948, pp 550-597.

4. Liebow, A. A., Warren, S., and de Coursey, E., *Am. J. Path.,* 1949, v25, 953.

5. Warren, S., *Arch. Path.,* 1943, v35, 121.

6. Tullis, J. L., *Am. J. Path.,* 1949, v25, 829.

7. Eschenbrenner, A., and Miller, E., *Arch. Path.,* 1950, v50, 736.

8. Bourg, R., *Arch. de biol.,* 1931, v41, 245.

9. Johnston, R. L., *Endocrinol.,* 1934, v18, 123.

10. Heald, A. H., Beard, C., and Lyons, W. R., *Am. J. Roentgenol.,* 1939, v41, 448.

Received April 24, 1953. P.S.E.B.M., 1953, v83.

Chromatographic Separation of Heparin and Chondroitin Sulfate.* (20327)

GRACE P. KERBY.
From the Department of Medicine, Duke University School of Medicine, Durham, N. C.

In a recent communication, Rienitis(1) has contributed interesting and useful data concerning the electrophoresis of acid mucopolysaccharides on filter paper. Certain mixtures of acid mucopolysaccharides have been resolved by the method, but the separation of heparin and chondroitin sulfate was not accomplished, due to the slightly different mobilities of the substances. It is of interest to note that such separation can be achieved chromatographically. The writer's current investigations are concerned with acid mucopolysaccharides as found in biological materials of human and animal source. In the course of these studies, chromatographic technics have been used, the filter paper strips (Whatman No. 1, 44 cm) being stained by a method developed in this laboratory and similar to that used by Rienitis. The accompanying photograph (Fig. 1) shows the distinct separation of a mixture of chondroitin sulfate and heparin. The ascending chromatogram was developed with 25% propanol in M/15 phosphate buffer at pH 6.4 for 33 hours at 4°C. Heparin Sodium (Liquaemin; Organon, Inc.) and Chondroitin Sulfate (General Biochemicals, Inc.) were used in the present study. Results of the application of this technic in the investigation of biological materials will be incorporated in later reports.

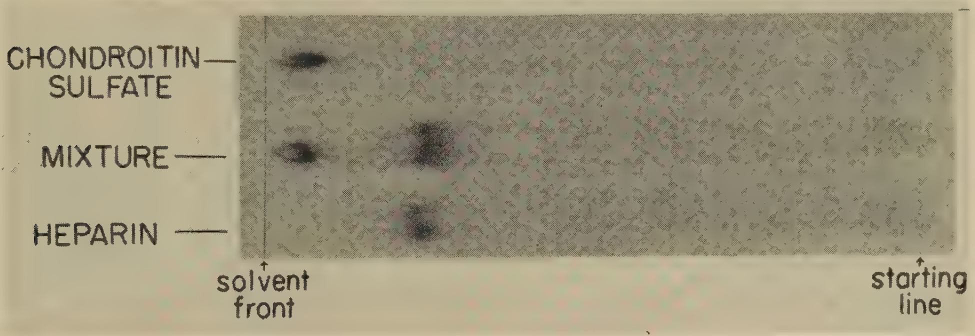


FIG. 1. Paper chromatographic separation of chondroitin sulfate and heparin.

---

* This work was supported in part by a grant from the National Microbiological Institute of the National Institutes of Health of the Public Health Service and by the Baxter Laboratories, Inc.

1. Rienitis, K. G., *Biochem. J.*, 1953, v53, 79.

Received April 27, 1953. P.S.E.B.M., 1953, v83.

Study of the Effect of Hematoporphyrin on Hypophyseal-Adrenal Relationships.* (20328)

George C. Peck, H. Patterson Mack, and Frank H. J. Figge.

From the Department of Anatomy, University of Maryland, School of Medicine, Baltimore.

It had been shown(1) that porphyrins injected into ovariectomized mice produced a series of estrous reactions, which reoccurred after the porphyrin injections were discontinued. It had also been shown that injection of porphyrins into amphibians caused a release of the melanophore-expanding hormone from the intermediate lobe of the hypophysis(2). It was, therefore, concluded that porphyrins had a stimulatory effect on the hypophysis and that the reactions following porphyrin injections were related to the secondary stimulation of the adrenal cortex, gonads, or melanophore system(3). When one of us attempted to induce estrous reactions by injecting porphyrin into ovariectomized mice, vaginal smears indicative of pseudo-estrous were obtained, but the results were not considered conclusive. Other methods to test the possible stimulatory influence of porphyrins on the hypophysis were then devised. In order to study any potential hypophyseal stimulator, the degree of specificity of response of the hypophysis should be evaluated. At the time that ACTH and Cortisone became popular therapeutic agents, it seemed desirable to devise a method of stimulating the hypophysis to produce greater amounts of ACTH, and it was hoped that hematoporphyrin would give rise to such specific stimulation.

The hypothesis involved in these experiments was also based on the observations of Nelson(4), who demonstrated that Cortisone had a definite inhibitory effect on anaphylaxis in the mouse. Since ACTH stimulates the adrenal cortex to elaborate Cortisone and Cortisone-like compounds, it was postulated that if porphyrin stimulated hypophyseal ACTH output, it might thus have an indirect Cortisone-like influence on anaphylaxis in the mouse. These experiments, therefore, involved studies on the influence of porphyrins, ACTH, Cortisone, and stress on anaphylaxis in the mouse. It will be shown that Cortisone did indeed prevent death from anaphylactic shock in the mouse(4) and that high doses of porphyrin also protected mice from anaphylactic shock. ACTH and low doses of porphyrin and various forms of stress either had no protective effect or increased the incidence of death from shocking doses of horse serum.

Methods and materials. The 446 mice used in these experiments were of the LCS_a,† Strong A, and C_3H strains. They were sensitized by injecting 1 cc of neutral undiluted horse serum‡ intraperitoneally every second day until a total of 4 cc had been administered to each mouse. This procedure is essentially the same as the method used by Mayer and Brousseau(5). After a sensitization period of 2 weeks to 2 months, each mouse received 1 cc of the same antigen by a rapid injection into the tail vein. The groups of mice were divided into control and experimental groups. The controls received no injections in the interim prior to the shocking dose of horse serum, except a few groups that received injections of physiological saline. Mice in the experimental groups received either hematoporphyrin, aqueous ACTH, Cortisone, or stress treatment 4-48 hours before the shocking dose of horse serum. The hematoporphyrin was given intraperitoneally; the ACTH and Cortisone intramuscularly. The dosages given to the various groups and the results are indicated in Table I. The 4 and 6 mg total doses of hematoporphyrin were divided into individual doses of 2 mg each, administered 24 and 4 or 48, 24, and 4 hours before the administration of a shocking dose of horse serum. When a sensitized control mouse was given 1 cc of horse serum by tail vein injection

---

* This work was supported by grants from the Anna Fuller Fund, the American Cancer Society, Maryland Division, and the National Institute of Health of the U. S. Public Health Service.

† The LCS_a strain was developed in this laboratory by crossing a CBAN with the Strong A mouse.

‡ Wyeth (without preservative)

TABLE I. Influence of Various Agents and Procedures on Survival of Sensitized Mice Injected with Shocking Doses of Horse Serum.

Drug inj.	Dose, mg	No. mice	No. survived	% survived	Difference from control	S.E., %	Difference/ S.E. of difference
Controls		148	38	26		3.6	
Hematoporphyrin	.6– 2	45	3	6.6	—20	3.7	3.9
”	4 – 6	86	39	45.3	+19	5.4	2.9
ACTH	2 –10	89	25	28	+ 2	4.7	.3
Cortisone	3	41	41	100	+74	0	20
Stress							
Phenol		19	0	0	—26	0	7.2
Air jet		18	1	5.5	—21	5.1	3.3

after a normal sensitizing period, the mouse usually died. The criterion for a protective effect of any treatment was simply based on whether a significantly greater percentage of the mice survived such a shocking dose of horse serum.

Observations and results. The number of mice in each group and the treatment received are shown in Table I. This also gives the number of mice that survived such treatment and the percentage of survival along with the standard error and an estimate of the significance of the differences observed. In all, 148 control mice were sensitized, and 38 of these survived a shocking dose of 1 cc of horse serum. This is a 26% survival. Forty-five mice were injected with low dosages of hematoporphyrin (0.6 mg to 2 mg). Only 3 of these survived the shocking dose of horse serum. This is a 6% survival. The difference between this and the control group was fairly large (20%) and appeared to be statistically significant.

When mice were injected with 4-6 mg of hematoporphyrin in divided doses 2 days before they received the shocking dose, a total of 39 (45%) of the 86 mice survived. This differs from the control group by 19% and involved a relatively large number of animals. Since this difference was 2.9 times the standard error of the difference, it is felt that high doses of porphyrin exerted a protective influence. The per cent survival of the mice receiving 4 and 6 mg of hematoporphyrin were so nearly the same that they were grouped together.

Eighty-nine of the sensitized mice were injected with aqueous ACTH (2-10 mg); one half of the dose was given 24 hours; the other half 4-6 hours before the administration of a shocking dose of horse serum. Twenty-five of the 89 mice or 28% survived. This is only 2% more survivors than in the control group. It was concluded that ACTH did not protect mice from anaphylactic shock.

In contrast, the protective effect of Cortisone was most striking. Forty-one of the sensitized mice were injected with 3 mg of Cortisone 20 hours before the intravenous injection of horse serum. All of the 41 mice survived. The difference between that and the control group was 74% or 20 times the standard error of the difference and is, therefore, highly significant.

As indicated in the table, 2 types of stress were used in the attempt to evaluate the influence of stress reactions on anaphylactic shock. Nineteen of the mice were injected with 0.5 cc and 0.25 cc of 0.5% phenol 24 and 4 hours before the shocking dose of horse serum. None of these mice survived. Eighteen of the mice were subjected to an air jet for 2 minutes, 24 and 4 hours prior to the injection of a shocking dose of horse serum. Only 1 mouse survived. At the time when the shocking dose was administered, the physical appearance and behavior of the mice in both the phenol-treated and air jet-stressed groups was normal. Neither of these procedures protected mice from anaphylactic shock, but on the contrary, had an adverse effect which was found to be statistically significant.

In studying the effect of stress or alarm reactions on anaphylactic shock in egg-albumin-sensitized guinea pigs, Leger, Leith, and Rose(6) found that mild stress protected the animals from anaphylactic shock. However, if severe stress and alarm reactions were elicited, and a shocking dose of egg albumin was given a few hours later, but before the alarm reaction had subsided, it actually aggravated the anaphylaxis. This may possibly explain some of our low survivals in the group of mice stressed with phenol injections and air jets.

Summary and conclusions. 1. Four hundred and forty-six mice were sensitized to horse serum, and 295 of these were treated with ACTH, Cortisone, hematoporphyrin, phenol, and air jets, in order to compare their influence on the survival of these mice when given a shocking dose of horse serum. 2. Twenty-six percent or 38 of 148 control mice sensitized to horse serum survived. 3. One hundred percent (41) of the horse serum-sensitized mice survived a shocking dose when treated with 3 mg of Cortisone 24 hours prior to the injection of 1 cc of horse serum. 4. ACTH failed to protect sensitized mice from shocking doses of horse serum, as did low doses of hematoporphyrin and stress procedures. Both of the latter procedures appeared to increase the incidence of death from anaphylaxis. 5. Hematoporphyrin in doses of 4-6 mg protected mice from shocking doses of horse serum, since a significantly higher percentage (45%) of the mice survived. 6. While our evidence indicates that high doses of hematoporphyrin protect mice from anaphylactic shock, no conclusions can be drawn concerning the hypothesis that porphyrins stimulate the hypophysis, because ACTH failed to exhibit any influence on anaphylaxis.

1. Rodewald, W., *Arch. Exp. Path. and Pharmacol.*, 1939, v194, 76.

2. Hinsberg, K., and Rodewald, W., *Arch. Exp. Path. and Pharmacol.*, 1938, v191, 1.

3. Figge, Frank H. J., Unpublished data.

4. Nelson, Carl A., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 181.

5. Mayer, Rudolf L., and Brousseau, Dorothy, PROC. SOC. EXP. BIOL. AND MED., 1946, v63, 187.

6. Leger, Jacques, Leith, W., and Rose, Bram, PROC. SOC. EXP. BIOL. AND MED., 1948, v69, 465.

Received May 6, 1953. P.S.E.B.M., 1953, v83.

Effects of X-Irradiation and Cysteine Injection on Enzyme Systems of Rat Tissues.* (20329)

MARIE A. FISCHER, ELLEN PURVIS COULTER, AND MICHAEL J. COSTELLO.
(Introduced by W. S. McEllroy.)

From the Department of Physiological Chemistry, School of Medicine, University of Pittsburgh.

Barron and his co-workers(1) reported that purified sulfhydryl-containing enzymes are inactivated by small amounts of x-rays and that the activities can partially be restored by glutathione. In the rat the oxidations of substrates requiring sulfhydryl-enzymes were shown to be diminished in all tissues, except the thymus, as a result of whole body x-irradiation(2) and it was demonstrated by Patt, Tyree, Staube and Smith(3) that cysteine or glutathione, administered prior to exposure, increased the number of animals able to survive a lethal dose of x-rays. Ludewig and Chanutin(4) found no change in the activity of rat liver catalase and certain other non-thiol enzymes but Feinstein, Butler and Hendley(5) demonstrated that a reduction in liver catalase activity follows the exposure of the whole mouse to x-rays. Barron(2) found that the oxidation of added succinate by kidney slices was depressed as a result of x-ray treatment, but LeMay(6) failed to find a decrease in the succinoxidase or cytochrome oxidase activities of kidney homogenates or slices. Citrate synthesis in the spleen, thymus, ileum, pancreas and testes of the rat is depressed by lethal doses of x-ray(7) but the activities of succinoxidase, adenosinetriphosphatase, and other sulfhydryl enzymes of several tissues were unaffected. More recently Ashwell and Hickman(8) showed that whole body irradiation does not affect spleen

---

* This work was supported by the Atomic Energy Commission.

succinoxidase but increased the spleen ATP-ase of the mouse.

In the present experiments the activities of two sulfhydryl enzymes (succinoxidase and adenosinetriphosphatase) and two non-sulfhydryl enzymes (lactic dehydrogenase and cytochrome oxidase) were determined in the kidneys, livers and spleens of untreated and cysteine-injected rats all of which had been exposed to a lethal dose of x-rays. The data show that spleen succinoxidase activity was depressed by x-ray treatment and that the decrease was less pronounced if the animals were injected with l-cysteine either before or after exposure to the rays.

Methods. Female Carworth Farms rats weighing 150 to 200 g were used in these experiments. The irradiated animals received 800 r total body x-radiation (200 kv, 20 m.a. with 0.5 mm Cu and 1 mm Al filters) at a rate of 18.5 r/min. When l-cysteine was administered an intraperitoneal injection of 875 mg/kg of body weight was given. A 15% cysteine solution, neutralized to pH 7.2 was prepared immediately before use. In each experiment 4 similarly treated animals were killed by decapitation and their tissues were pooled in order to have sufficient quantities of each tissue to assay for the 4 enzymes. The livers and spleens were analyzed immediately for lactic dehydrogenase(9), succinoxidase and cytochrome oxidase(10). The kidneys, however, were stored for one hour in the freezing compartment of the refrigerator before they were homogenized for assay. Homogenates were prepared in a Potter-Elvehjem type homogenizer and the analyses were made in the Warburg apparatus at 37° with air as a gas phase. In a later series of experiments succinoxidase and cytochrome oxidase activities were measured on the separate spleens of individual animals. Since these analyses were made at 38°C, temperature will be recorded in the tables of data. ATP-ase activities of the liver, spleen and kidney tissues were determined by the method of DuBois and Potter(11) as soon as possible after the animals were sacrificed. The amount of inorganic phosphorus liberated from ATP in 15 minutes at 37°C was measured by the Fiske and Subbarow procedure.

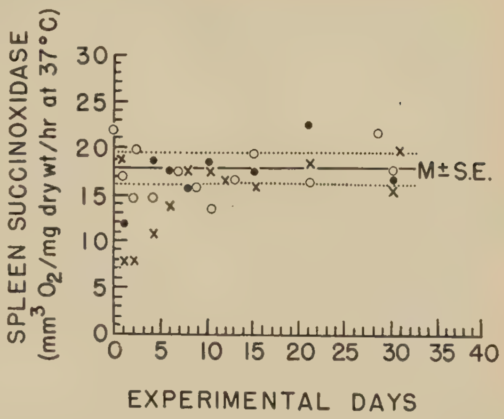


FIG. 1. Changes in the spleen succinoxidase activity of X-irradiated (×), cysteine-inj. and irradiated (○) and of cysteine inj. rats (●). The solid line represents the mean value of the spleen succinoxidase activities of untreated controls and the dotted lines, twice the stand. error of this mean.

Results. The activities of adenosinetriphosphatase, succinoxidase, cytochrome oxidase and lactic dehydrogenase from the kidney were not affected by whole body x-irradiation. Furthermore, the injection of l-cysteine into normal animals or pretreatment of irradiated animals with cysteine had no significant effect on the activities of these 4 enzymes. Similar results were obtained for the enzymes of liver.

A survey experiment was also carried out on the enzymes of the spleen. With one exception, spleen succinoxidase, no significant difference could be shown to exist between the activities of the spleen enzymes of the normal and treated groups of animals. Plots of the data were made to determine whether a temporary change in the enzyme activities had occurred. In the case of ATP-ase, cytochrome oxidase and lactic dehydrogenase, the values for each experimental day were randomly distributed. The succinoxidase values from spleens of irradiated animals, however, were depressed during the first week after irradiation (Fig. 1) and minimal values were reached about the second experimental day. This reduction in succinoxidase activity was prevented by administration of l-cysteine prior to the irradiation, although there is no evidence that l-cysteine administered to unexposed animals affects the enzyme in any way. Because of these findings it seemed worthwhile

TABLE I. Effects of X-Irradiation and Pre- and Post-Irradiation Injections of l-Cysteine on Spleen Succinoxidase.*

	No. of assays	Spleen succinoxidase†	Spleen wt, g†	30-day survival, %‡
Control	30	19.2 ± .5	.403	100
Irradiated	29	14.9 ± .6	.195	0
Cysteine inj. + irradiated	29	18.0 ± .5	.241	60
Irradiated + cysteine inj.	30	18.3 ± .8	.219	10
Cysteine inj.	22	18.7 ± .4	.437	100

* Values expressed as mm^3 O_2/mg dry wt/hr at 38°C.

† Figures are averages of determinations made from 2 hr through 4 days after treatment. Stand. errors of the mean are recorded for the enzyme assays.

‡ The 30-day survival was determined in a separate experiment. Each group consisted initially of 10 rats.

to reinvestigate the succinoxidase activities but to limit the study to the first four experimental days. Animals were therefore sacrificed either 2 hours, 1, 2, 3, or 4 days after treatment and succinoxidase of the individual spleens were determined. Spleens from normal control animals were assayed in parallel experiments and the results given in Table I show that spleen succinoxidase was significantly (t = 5.5) depressed following exposure to x-rays. Pretreatment with l-cysteine prevents this decrease in succinoxidase and increases the number of survivors. The two effects are unrelated for the same quantity of l-cysteine, given to animals after they had been exposed to the x-rays (Table I), gave practically no protection as measured by survival or spleen weight even though it did prevent to a large extent the decrease in spleen succinoxidase activity.

Discussion. Ashwell and Hickman found irradiation had no effect on the succinoxidase of mouse spleen(8). They averaged data over a comparatively long post-irradiation period. As shown in Fig. 1 to demonstrate the effect, determinations must be made within a few days after exposure. Ashwell and Hickman (8) reported a 4-fold increase in spleen ATPase but based their calculation of enzyme activity on the nitrogen or phosphorus content of the spleen whereas our activities were based on the dry weight of the tissue. On this basis we found no difference.

Conclusions. 1. Whole body x-irradiation has no effect on the adenosinetriphosphatase, cytochrome oxidase or lactic dehydrogenase activities of spleen, liver or kidney of rats. 2. Succinoxidase activity was reduced in the spleen of irradiated rats but remained normal in the liver and kidneys. 3. The *in vivo* inhibition of spleen succinoxidase by x-rays could be prevented by either pre- or post-irradiation injection of l-cysteine.

1. Barron, E. S. G., Dickman, S., Muntz, J. A., and Singer, T. S., *J. Gen. Physiol.*, 1949, v32, 537.
2. Declassified Document No. AECD-2316 U. S. Atomic Energy Commission, 1946.
3. Patt, H. M., Tyree, E. B., Straube, R. L., and Smith, D. E., *Science*, 1949, v110, 213.
4. Ludewig, S., and Chanutin, A., *Arch. Biochem.*, 1950, v29, 441.
5. Feinstein, R. N., Butler, C. L., and Hendley, D. D., *Science*, 1950, v111, 149.
6. LeMay, M., PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 337.
7. DuBois, K. P., Cochran, K. W., and Doull, J., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 422.
8. Ashwell, G., and Hickman, J., PROC. SOC. EXP. BIOL. AND MED., 1952, v80, 407.
9. Vestling, C. S., and Knoepfelmacher, A. A., *J. Biol. Chem.*, 1950, v183, 63.
10. Schneider, W. C., and Potter, V. R., *J. Biol. Chem.*, 1943, v149, 217.
11. DuBois, K. P., and Potter, V. R., *J. Biol. Chem.*, 1943, v150, 185.

Received May 8, 1953. P.S.E.B.M., 1953, v83.

Effect of Crystalline Trypsin on the Raw Soybean Growth Inhibitor. (20330)

H. J. ALMQUIST AND J. B. MERRITT.
From the Grange Company, Modesto, Calif.

In a previous report(1) we showed that trypsin (1-300) fed at 0.15% of the diet neutralized most of the effect of the growth inhibitor present when one-fourth of the protein in the diet of the chick was provided as raw soybean meal. This relative amount of raw meal caused practically as much growth inhibition as did higher proportions. It has been indicated that the substance in crude trypsin, which is able to reverse the action of the soybean growth inhibitor in rat diets, is not trypsin but some associated impurity(2). In a critical test of this question we have fed crystalline lyophilized trypsin in chick diets containing raw soybean protein.

Methods. The procedure was the same as previously described(1). Chicks were carefully selected for uniform size and vitality and then divided equally into pens of 9 each.

Results. The results of 2 experiments are given in Table I. These show a distinct increase in weight gains when the crystalline trypsin was added.

Discussion. It is not certain that the amount of trypsin added was optimal, but this amount was clearly sufficient to demonstrate the anti-inhibitor activity of highly purified trypsin. We have shown that a low level of trypsin may exert no discernible effect, presumably because a low level may be completely inactivated in the presence of a surplus of the antitrypsin known to be present in the raw meal(1). Enough trypsin must be added to neutralize the antitrypsin to the extent that proteolytic enzymes secreted by the animal may be allowed a moderate to complete freedom of action.

TABLE I. Effect of Crystalline Trypsin on the Growth Inhibition Caused by 5% Raw Soybean Protein in Chick Diets.*

Trypsin† added to 5% raw protein diet, %	Avg wt of chicks, g	
	Exp. 1, 25 days	Exp. 2, 27 days
.00	191.4	206.6
.05	223.9	244.3

* Diets contained a total 20% soybean protein plus 0.3% methionine.

† Worthington Biochemical Sales Co., crystallized, lyophilized trypsin.

1. Almquist, H. J., and Merritt, J. B., PROC. SOC. EXP. BIOL. AND MED., 1952, v79, 277.

2. Borchers, R., and Ackerson, C. W., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 81.

Received May 11, 1953. P.S.E.B.M., 1953, v83.

Application of Thermistor to Measurement of Subcutaneous Temperatures During Hydrothermal Injury in the Rat.* (20331)

RICHARD S. GRAY AND A. E. AXELROD. (Introduced by Alan R. Moritz.)
From the Institute of Pathology and the Department of Biochemistry, Medical School, Western Reserve University, Cleveland, O.

During the course of experiments conducted in this laboratory on the biochemical changes in skin resulting from hydrothermal exposure of the anesthetized rat(1), it became necessary to obtain a continuous recording of the subcutaneous temperatures in areas of skin exposed at 50°-70°C. The present paper describes an apparatus incorporating a thermistor as the temperature-sensing element which has been used successfully during the past year for this purpose.

Development of the apparatus. In developing a suitable apparatus, it was recognized that the temperature-sensing element which was to be placed under the skin must necessarily be very small and must possess a very low thermal capacity in order to permit an immediate and accurate recording of the temperature changes (30°-70°C) in the surrounding medium. Since a continuous temperature recording was desired, it was imperative that a recording instrument be incorporated.

---

* This work performed under contract between the U. S. Atomic Energy Commission and Western Reserve University.

The application of the thermocouple to the present problem was given first consideration. Extremely small needle thermocouples in which the thermocouple junction is placed in the end of a hypodermic needle can be constructed from commercially available thermocouple wire and stainless steel hypodermic tubing(2-4). Thermocouples, however, have their disadvantages when used for low temperature measurements. The signal resulting from a temperature change of a few degrees is very small, of the order of microvolts. The leads connecting the junctions and the indicating instrument must be of the junction metals. Any inhomogeneity, either of composition or structure, in the leads will produce spurious potentials if subjected to a temperature gradient. Finally, there is the necessity for maintaining a constant reference junction temperature. The accuracy of the thermocouple is no better than that of the temperature of the reference junction.

These drawbacks to the thermocouple directed our attention to the application of other devices of electrical thermometry. For many years the changes in resistance of certain materials, *e.g.*, platinum wire, with changes in temperature have been used as the principle of many thermometric devices. In 1945, a new circuit element, the thermistor, was introduced. The thermistor is a resistor made of a semi-conductor with a negative thermal coefficient of resistance as high as 4.7% per degree C(5). Thermistors are being used extensively for current control and temperature-sensing elements. They are made in a large variety of sizes and shapes. One of the smallest sizes currently available is a glass-covered bead 0.015″ in diameter. Its resistance at 20°C is 2000 ohms, which falls to 900 ohms at 50°C. A temperature change from 20° to 50°C will produce a voltage change of 0.110 volt with only 0.0001 ampere flowing through the thermistor. In contrast, an iron-constantan thermocouple will produce only 0.0015 volt with the same temperature differential. Thus, with the thermistor it is possible to use a much less sensitive indicating element or to eliminate the amplification used with the thermocouples. The meter zero may be set at any temperature up to 150°C by adjusting the balance of the bridge. The necessity for maintaining a reference temperature is obviated. The resistance of the thermistor is high enough that normal changes in the resistance of the leads may be neglected. Any instability resulting from resistance changes of the other resistors in the circuit can be compensated by a variable resistor.

Drummeter and Fastie(6) have successfully applied the thermistor to the measurement of blood temperature and their experiences led us to attempt the adaptation of the thermistor to our needs. Thermistors have also been employed to measure the very small temperature changes occurring in an enzyme-catalyzed reaction(7). Western Electric No. 27A thermistor glass-covered beads, 0.015″ in diameter and 0.020″ long, with both leads coming from the same end, are employed in the present study. The leads are 0.001″ platinum wire and are ⅜″ long. Thermistors with longer leads are available commercially.[†] To permit insertion under the skin, the thermistor bead is placed in the end of a 4″ piece of 24-gauge stainless steel needle tubing (Fig. 1). The lumen at one end of the needle is enlarged with a No. 78 drill (0.016″) to receive the thermistor. The needle is then sharpened in the usual manner and the back end of the bevel extended as shown in Fig. 1. After honing the point, removing burrs from the edges and washing to remove abrasive, the point is ready to receive the bead. The lead wires should be coated twice with insulating varnish thinned with benzene. The bead is covered with cement to insulate it from the needle and worked into place, leaving the upper surface of the bead exposed. The cement should not contain a solvent which can

---

[†] Victory Engineering Corp., Newark, N. J.

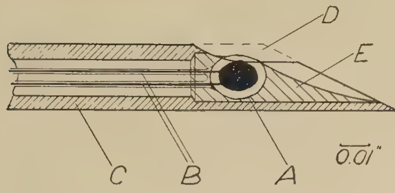


FIG. 1. Diagram of the thermistor needle-type thermometer. A, glass-covered thermistor bead; B, insulated leads; C, needle tubing; D, portion of the needle wall ground off to expose the top of the bead; E, cement holding the bead in position.

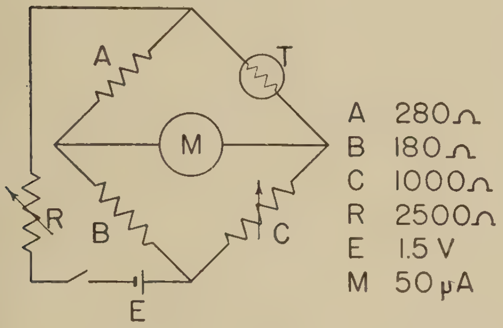


FIG. 2. Wheatstone bridge circuit used with W.E. thermistor No. 27A for measuring temperatures in the range from 35° to 60°C. A and B are selected to give a linear relationship between temperature of the thermistor T and deflection of the meter M. C and R are adjusted for balance and desired sensitivity, respectively.

dissolve the insulation from the wires. A handle may be constructed on the other end of the needle and provision made to connect the wires which go to the bridge.

The relationship between temperature and resistance of a thermistor is non-linear as is the off-balance current of a Wheatstone bridge with respect to changes in thermistor resistance. Using a 1:1:1:1 bridge circuit, these non-linearities compensate adequately for a range of 10°C, yielding a linear meter-temperature curve. Drummeter and Fastie(6) obtained linearity over a 20°C range by using a zero-center meter and measuring 10°C above and below the bridge balancing point. In order to obtain linearity over a greater temperature range with the left-center recording meter available to us, it became necessary to modify the resistance values as shown in Fig. 2. The bridge is balanced at 35°C by means of the variable resistor at C. With the thermistor at 60°C, R is adjusted to give full scale deflection. With the zero and full scale readings set as above, the shape of the meter-temperature curve will change with changes in A:T. Further modification may be effected by changing the ratio A:B and the resistance of the meter M. Increasing A:B to 1:10 or more will improve the linearity of meter vs. resistance without necessarily improving the linearity of the meter vs. temperature curve(8). For calibration purposes, the thermistor resistance was measured at a number of known temperatures over the desired temperature range (25°-70°C) by immersing the thermistor in a stirred water bath whose temperature was determined with a National Bureau of Standards calibrated thermometer. In order to facilitate the checking of the linearity of the meter and its calibration, these resistance values could be substituted for the thermistor by means of a decade resistance box.

With the thermistor utilized in our apparatus, the resistance values shown in Fig. 2 give a linear meter deflection from 35° to 60°C within 0.05°C. When a chart record is desired, the meter is paralleled with a G-E photoelectric self-balancing potentiometer millivoltmeter with a sensitivity of 50 millivolts full scale and R adjusted for full scale recording meter deflection. For a more accurate fast response of the millivoltmeter, the microammeter should be replaced with a resistance whose value is that of the resistance of the meter.

The manufacturing tolerances on thermistors are ± 25%, and if more than one thermistor is to be used with one bridge, compensation may be added to the individual thermistor units by the method described by L. J. Anderson(9). This will match several thermistors at 2 points on the temperature-resistance curve at a slight sacrifice of sensitivity.

The needle-type thermistor thermometer described above has been used extensively in our laboratory to obtain a continuous recording of abdominal subcutaneous temperatures in areas of skin subjected to temperatures ranging from 50° to 70°C for varying periods of time(1). The needle was inserted under

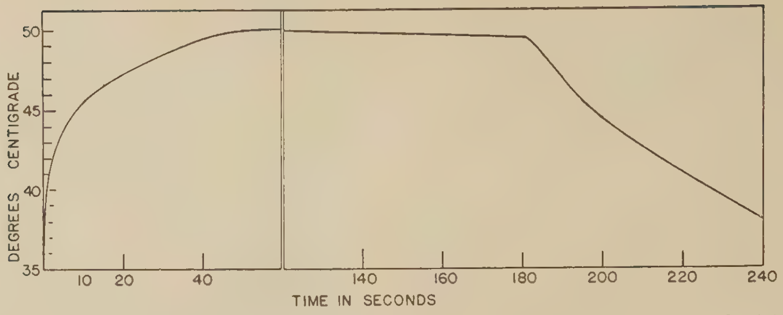


FIG. 3. Subcutaneous temperatures in a rat exposed to circulating water at 55°C. The rat was placed in water bath at T = 0 sec. and removed at T = 180 sec.

the skin before exposure, its position being verified by dissection following the thermal episode. A typical example of a subcutaneous temperature curve is shown in Fig. 3.

Summary. A simple and sensitive thermoelectric device for the measurement of temperature has been described which incorporates the thermistor as the temperature-sensing element. As a needle-type thermometer, this apparatus has been used extensively in our laboratory to obtain continuous recordings of abdominal subcutaneous temperatures attained during the production of hydrothermal burns in rats. On the basis of the many advantages inherent in this apparatus, its further utilization for temperature measurements in biological studies is advocated.

---

1. Axelrod, A. E., and Martin, C. J., PROC. SOC. EXP. BIOL. AND MED., (submitted).

2. Henriques, F. C., *R. S. I.*, 1947, v18, 637.

3. Sheard, C., *Am. J. Clin. Path.*, 1931, v1, 209.

4. Mendelssohn, K., and Rossiter, R. J., *Quart. J. Exp. Physiol.*, 1944, v32, 301.

5. Dowell, K. P., *Electrical Manufacturing*, August, 1948.

6. Drummeter, L. F., Jr., and Fastie, W. G., *Science*, 1947, v105, 73.

7. Bauer, C. R., and Gemmill, C. L., *Arch. Biochem. and Biophys.*, 1952, v35, 111.

8. Huss, P. O., Paper presented at Conference of the Instrument Society of America, Cleveland, Ohio, September, 1952.

9. Anderson, L. J., *Bull. Am. Meteorol. Soc.*, 1949, v30, 192.

Received May 12, 1953. P.S.E.B.M., 1953, v83.

Resistance to Folic Acid Analogues in a Strain of *Streptococcus faecalis*.*

(20332)

CHARLES A. NICHOL,[†‡] SIGMUND F. ZAKRZEWSKI,[‡] AND ARNOLD D. WELCH.[‡]

From the Department of Pharmacology, School of Medicine, Western Reserve University, Cleveland, O.

The possibility that organisms which are resistant to certain antagonists of folic acid possess or develop the ability to convert such analogues to metabolically active compounds has formed an attractive hypothesis. Aminopterin (4-amino-pteroylglutamic acid) is readi-

---

* This investigation was supported, in part, by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service, and, in part, by a grant from the Lederle Laboratories Division of the American Cyanamid Co.

[†] Scholar in Cancer Research of the American Cancer Society.

[‡] Present address: Department of Pharmacology, Yale University School of Medicine.

[§] We are indebted to Dr. Joseph H. Burchenal and Dr. Dorris J. Hutchison for cultures of the antagonist-resistant strains of *S. faecalis* and *Leuconostoc citrovorum*.

ly converted to pteroylglutamic acid (PGA) by alkaline hydrolysis(1). Kidder *et al.*(2) observed that Aminopterin and Methopterin (10-methyl PGA) promoted the growth of a species of *Tetrahymena* and suggested that this organism possesses enzymes capable of deaminating and demethylating these antagonists. Hutchison and Burchenal(3) also suggested that an antagonist-resistant strain of *Streptococcus faecalis*(4) utilizes in a similar manner these and related analogues for growth. In preliminary experiments designed to test this concept by measurement of precursors of citrovorum factor (CF) which might be derived from the antagonists, technics were developed for the separation of 4-amino and 4-hydroxy pteridines(5).

It has been found, employing paper chromatographic technics, that the folic acid-antagonists which are presently available contain sufficient PGA and pteroic acid to account for the apparent "utilization" of the analogues by *S. faecalis*. The remarkable increase in the resistance to certain folic acid antagonists which had been induced in the strain of *S. faecalis* studied(4) can be explained, in part, by its more effective utilization of PGA and pteroic acid, as shown in these studies. Similar observations were reported recently by Hutchison and Burchenal(6). That other phenomena are involved in the development of resistance will be discussed in detail in a subsequent communication.

Methods. Microbial assays—Streptococcus faecalis (ATCC 8043) was used for the microbial determination of folic acid-like activity, as described in the AOAC Methods of Analysis (7). An antagonist-resistant strain of *S. faecalis*§(4), designated as 8043AR, was used similarly for the assay of folic acid-like activity in the presence of the antagonists. In each case, a sample of pteroylglutamic acid was used as a reference standard. Citrovorum factor (CF) was determined using *Leuconostoc citrovorum* (ATCC 8081) grown in the medium described by Sauberlich(8). An antagonist-resistant strain of *Leuconostoc citrovorum*§(9), designated as 8081AR, was used in the same manner for the assay of CF in the presence of high concentrations of the antagonists. Leucovorin (synthetic CF) was used as a reference standard. The activity of the samples is expressed on the basis of the natural form of the free acid, since the naturally-occurring form of CF appears to be twice as active as leucovorin. The basis for this statement has been discussed elsewhere(10).

Bioautographic technic. The distribution of the compounds on paper chromatograms was followed by means of a modification of previously described bioautographic technics (11,12). Solid medium (folic acid-assay medium containing 1% agar) was inoculated with a strain of *S. faecalis* immediately before the medium was poured into suitable trays. The paper strips were placed on the surface of the agar for 10 min; the trays then were placed in a 37° incubator. The zones of growth or inhibition were visible within 6 hours, but recordings were made after an 18-hour period. Growth-promoting compounds were readily located when PGA was omitted from the medium. When PGA was included in the medium at a level of 10 mμg/ml, the agar was clouded by growth except where inhibitory compounds caused clear zones. With a smaller amount of PGA in the medium, 1 mμg/ml, so that only limited growth resulted, both inhibitory and growth-promoting compounds could be readily located on a single bioautograph. By addition to the medium of 2,3,5-triphenyl tetrazolium chloride (200 μg per ml), which is reduced to an insoluble red compound by the growing cells, the visibility of areas of faint growth was improved and better contrast for photographic records was obtained. However, zones of growth caused by 0.5 mμg of PGA per strip were clearly visible without this aid. A convenient inoculum was prepared by lyophilization of the washed bacterial cells in a lactose suspending-medium(13). This preparation retained activity for a period of approximately 3 months when stored at room temperature in a desiccator (1 mg of the lyophilized cells was adequate for 100 ml of medium). Chromatograms, using strips or sheets of Whatman No. 1 paper, were developed by descending solvent (0.1 *M* phosphate buffer, pH 7.0) which moved 44 to 48 cm in 4 hours. When dry, the strips (1″ in width) were cut in half lengthwise. In order to locate both inhibitory and

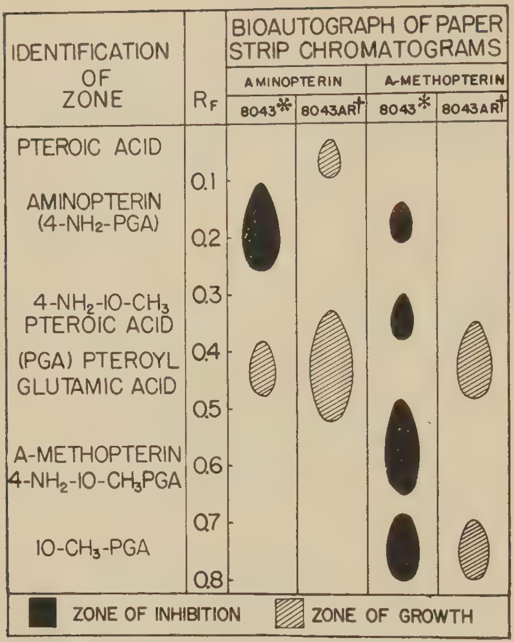

IDENTIFICATION OF ZONE
R_F
BIOAUTOGRAPH OF PAPER STRIP CHROMATOGRAMS
AMINOPTERIN
A-METHOPTERIN
8043*
8043AR†
8043*
8043AR†
PTEROIC ACID
AMINOPTERIN (4-NH_2-PGA)
4-NH_2-10-CH_3 PTEROIC ACID
(PGA) PTEROYL GLUTAMIC ACID
A-METHOPTERIN 4-NH_2-10-CH_3PGA
10-CH_3-PGA
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
■ ZONE OF INHIBITION
▨ ZONE OF GROWTH

FIG. 1. Comparison of response of antagonist-sensitive (8043) and antagonist-resistant (8043-AR) strains of *S. faecalis* to Aminopterin and A-methopterin.
* Antagonist-sensitive strain of *S. faecalis.*
† " -resistant " " " " .

growth-promoting compounds, one half of each paper strip was placed on solid medium which contained PGA, the other half on medium without PGA. *Resting cell experiments.* Cells of strains 8043 and 8043AR were grown in a medium containing PGA (1 mμg/ml), harvested after 16 hours, washed twice in saline (0.9%), and the weight of the packed cells was recorded. The cell suspensions, in 0.1 *M* phosphate buffer of pH 6.5, were agitated constantly while incubating anaerobically at 37° with PGA or Aminopterin and the other additions indicated. At the end of the incubation period the samples were autoclaved at a pressure of 15 pounds for 30 min. The cells were spun down and the clear extract was appropriately diluted for microbial assay with *Leuconostoc citrovorum.*

Experimental and results. Bioautographs of Aminopterin and A-methopterin (4-amino-10-methyl-pteroylglutamic acid), in which the response of strains 8043 and 8043AR were compared, are shown in Fig. 1. With the antagonist-sensitive strain, large clear zones indicated the location of Aminopterin and A-methopterin. No effect was visible in the corresponding areas of the other half of each strip which was placed on medium containing the antagonist-resistant organism. Aminopterin contained two growth-promoting compounds with R_f values which corresponded to those of PGA and pteroic acid, respectively. The PGA which was present on one half of the strip consistently gave larger zones with strain 8043AR and the amount of pteroic acid which was adequate for the growth of strain 8043AR was insufficient to cause visible growth of strain 8043. A-methopterin was contaminated with 3 inhibitory compounds with R_f values which corresponded to Aminopterin, 4-amino-10-methyl-pteroic acid and 10-methyl-pteroylglutamic acid, respectively. Also, a growth-promoting compound was present which apparently was PGA. Growth of the antagonist-resistant organism was consistently observed in the area corresponding to 10-methyl-PGA; however, 2-dimensional chromatography showed that this zone contained PGA.

Tentative *identification of the contaminants* found in Aminopterin and A-methopterin, based on their R_f values, was made by comparison of the bioautographs shown in Fig. 2. Growth promoting compounds were observed in pteroylaspartic acid and in 4-amino-pteroylaspartic acid. Aminopterin-2 was obtained by elution with distilled water of the zone (R_f-0.12 to 0.29) obtained when Aminopterin-1, the initial material, was chromatographed on paper sheets. The amount of Aminopterin-2 was determined by microbial and fluorometric assays(5). Although the zones of Aminopterin and PGA were distinctly separate, only partial purification of Aminopterin was obtained in this way. A-methopterin-2 was obtained by elution of the A-methopterin zone (R_f-0.43 to 0.62) from the chromatogram of the initial material. A-methopterin-3 (Fig. 2) was derived from A-methopterin-2 in the same manner. Although the materials were protected from light and heat during these operations, to minimize degradation of the compounds, the twice-chromatographed A-methopterin-3 still contained detectable amounts of two contaminants. The antagonist-resistant strain of *S. faecalis* was used to study the growth-promoting activity of the analogues. The biological activity of the analogues (Fig. 2) cannot be expressed as a quantitative measure of the activity of a single compound, since most of the analogues studied contained pteroic acid as an active contaminant.

The requirement for CF was the same for each bacterial strain, but the resistant strain required much less PGA or pteroic acid than the sensitive strain (Table I). PGA and leucovorin were equally active for strain 8043, but PGA was more active than leucovorin for strain 8043AR (it should be noted that these findings take into account the circumstance that leucovorin appears to be composed of inert material to the extent of 50%) (10).

Cell suspensions of the antagonist-sensitive and antagonist-resistant strains of *S. faecalis* were incubated with PGA and the amount of CF formed was determined by microbial assay (Table II). Only an insignificant increase in the amount of CF formed by strain 8043 was obtained when the concentration of PGA in the suspending medium was greater than 0.5 μg/ml. However, with the resistant strain, the amount of CF formed increased as the concentration of PGA was increased; thus, at a PGA-level of 50 μg/ml this organism formed more than 200 times as much CF as the sensitive strain under the same conditions. In the same experiment, Aminopterin or A-methopterin in amounts of 0.1 μg per ml caused approximately 50% inhibition of the formation by strain 8043 of CF from PGA, while a similar degree of inhibition of the formation of CF from PGA by strain 8043AR required a 500-fold increase in the concentration of these antagonists.

The amounts of CF formed from the two samples of Aminopterin and from PGA are shown in Table III. The CF derived from Aminopterin-1 corresponded to approximately 10% of the amount added. The yield of CF from PGA was approximately 50%. Therefore, the precursors of CF in Aminopterin-1 can be estimated to be about 20% of the reagent material. The yield of CF from the

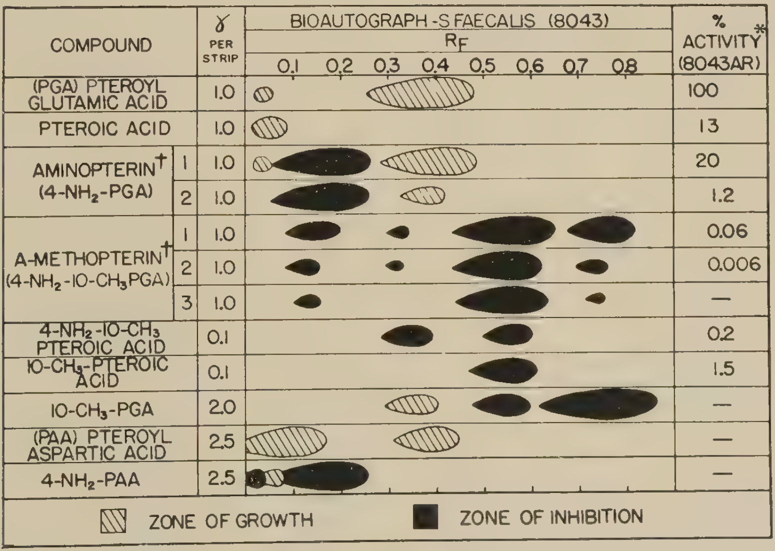


FIG. 2. Paper chromatograms and growth-promoting capacity of several analogues of pteroylglutamic acid.

---

* Comparison of growth-promoting capacity based upon microbial assay of analogues, using antagonist-resistant strain of *S. faecalis* (8043 AR). Large amounts of pteroic acid required to promote growth of antagonist-sensitive strain (8043) which was used for bioautographs shown here (see Fig. 1 and Table I).

† Aminopterin-2 obtained by elution of the Aminopterin zone of a chromatogram of Aminopterin-1, the commercial material. Similarly, A-methopterin-2 and -3 are, respectively, the once and twice chromatographed materials (see text for details).

partially purified Aminopterin-2 was exceedingly small.

Discussion. The presence of contaminants in various folic acid-antagonists has caused considerable confusion in the interpretation of the results of studies of resistance to these compounds. The antagonists which are presently available contain not only sufficient PGA and pteroic acid to account for the apparent utilization of the analogues by *S. faecalis*(3), but other inhibitory compounds may also be present, as found in A-methopterin. The growth-promoting capacity of Aminopterin (one-sixth that of PGA) reported for a species of *Tetrahymena*(2) actually corresponds closely to the amount of PGA found in these studies to be present in samples of the analogue. It will be of importance to determine the effect of the simultaneous presence of these growth-promoting compounds on the rate of development of resistance, since the resistant strain of *S. faecalis* utilized PGA and pteroic acid much more effectively than did the antagonist-sensitive strain.

TABLE I. Requirement for PGA, Pteroic Acid, and CF of Antagonist-Sensitive (8043) and Antagonist-Resistant (8043AR) Strains of *S. faecalis.*

Compound	Requirement for half-maximal growth* I Strain 8043	II Strain 8043AR	Approx. ratio I/II
	mμg/ml of assay medium		
Pteroylglutamic acid	.18	.06	3
Pteroic acid	7.0	.6	12
Citrovorum factor†	.16	.17	1

* Turbidimetric reading of growth in 10 ml of assay medium.

† A standard solution of leucovorin was used; activity is expressed in terms of naturally-occurring CF.

TABLE II. Formation of CF from PGA by Resting Cells of Antagonist-Sensitive (8043) and Antagonist-Resistant (8043AR) Strains of *S. faecalis.*

Additions to suspending medium* PGA, μg/ml	Ratio	CF formed by cell suspensions of *S. faecalis* Strain 8043	Strain 8043AR
	Antagonist:PGA	μg CF/25 mg cells, wet wt	
0	—	0	0
.5	—	.16	.20
5	—	.18	3.21
50	—	.20	44.4
	Aminopterin:PGA		
50	1:1	—	17.0
”	1:10	—	39.9
”	1:100	.038	42.3
”	1:250	.084	—
”	1:500	.11	—
”	1:1000	.15	—
	A-methopterin:PGA		
50	1:1	—	23.1
”	1:10	—	37.5
”	1:100	.025	37.0
”	1:250	.070	—
”	1:500	.11	—
”	1:1000	.14	—

* Cells (25 mg/ml, wet wt) incubated at 37° for 4 hr under an atmosphere of nitrogen (95%) and carbon dioxide (5%) in 2 ml of a medium consisting of Na_2HPO-KH_2PO_4 buffer (0.1 *M*, pH 6.5), glucose (0.02 *M*), sodium formate (0.01 *M*), sodium ascorbate (0.005 *M*) and PGA, Aminopterin or A-methopterin, as indicated. Preparation of samples for assay described in text.

TABLE III. Formation of Citrovorum Factor from Precursors in Commercial and Partially Purified Samples of Aminopterin.

Conc. of PGA or Aminopterin in suspending medium,* μg/ml	CF formed by cell suspensions of *S. faecalis* (8043AR) PGA	Aminopterin–1†	Aminopterin–2†
	μg CF/25 mg cells, wet wt		
0	0	0	0
1.5	4.76	.56	—
3	7.26	1.16	.05
6	12.2	2.53	.04
12	18.2	5.36	.03

* Cells (6.25 mg/ml, wet wt) incubated at 37° for 4 hr under an atmosphere of nitrogen (95%) and carbon dioxide (5%) in 4 ml of a medium consisting of Na_2HPO_4-KH_2PO_4 buffer (0.1 M, pH 6.5), glucose (0.02 *M*), sodium formate (0.01 *M*), sodium ascorbate (0.005 *M*) and PGA or Aminopterin as indicated. Preparation of samples for assay described in text.

† Aminopterin–1 refers to the commercial material, while Aminopterin–2 is that which had been purified by paper chromatography, described in text.

Instead of deaminating the 4-amino analogues of PGA, it appears from these studies that in the resistant cell the ability to absorb the analogues may have been reduced greatly. Thus, the formation of CF from PGA by resistant cells of *S. faecalis* is inhibited only by high concentrations of Aminopterin or A-methopterin, while in extracts of these cells, the same reaction is inhibited by amounts of these antagonists which also inhibit the formation of CF by the antagonist-sensitive cells or their extract(14). No detectable effect of these antagonists was observed on bioautographs obtained with the *intact* resistant organism: neither growth, indicating utilization of these compounds, nor inhibition, which might be anticipated if the compounds were able to exert their expected effect within the cells. Hutchison and Burchenal(9) reported that the requirement for CF of the antagonist-resistant strain of *Leuconostoc citrovorum* was the same as that of the parent strain and also, this organism, like the parent strain, was unable to utilize PGA effectively. Conceivably, the primary mechanism of resistance in *Leuconostoc citrovorum* involves an altered permeability to the antagonists, since a more efficient utilization of the factor required for growth does not appear to be implicated.

Paper chromatography is an aid to the identification and purification of these compounds but does not ensure either. In the system used, A-methopterin has the same R_f as 10-methyl-pteroic acid. The inhibitor at R_f 0.55, which was present in the sample of 10-methyl-PGA, was found, by the use of a different solvent, to migrate in the same manner as 10-methyl-pteroic acid. "Trailing" of compounds is frequently observed on paper chromatograms. Consequently, the presence of some PGA at the zone of Aminopterin was not surprising. However, twice chromatographed A-methopterin "carried" a slower moving inhibitor. Thus, it appears that these similar compounds have considerable ability to "carry" each other. A two-dimensional chromatogram of 10-methyl-PGA showed that the discreet zone of R_f 0.75 separated into two components, in the second dimension; one of these apparently was PGA. Although 10-methyl-PGA and 10-methyl-pteroic acid were apparently "utilized" by the resistant strain, as indicated by distinct zones of growth on the bioautographs, clarification of this possibility must await additional purification of the compounds.

Summary. Bioautographs of several 4-amino analogues of folic acid indicated the presence of contaminants which corresponded to the demethylated, deaminated, or pteroic acid analogues. All the antagonists studied contained sufficient pteroylglutamic acid or pteroic acid to account for their apparent utilization for growth by an antagonist-resistant strain of *Streptococcus faecalis*. This strain had a lower requirement for PGA and pteroic acid and a much greater capacity to convert PGA to CF than did the parent antagonist-sensitive strain. Significant inhibition of the formation of CF from PGA by the resistant cells was obtained only with very high concentrations of Aminopterin or A-methopterin.

We are indebted to the Lederle Laboratories Division and the Calco Chemical Division of the American Cyanamid Co. for supplies of leucovorin, and for pteroylglutamic acid, and its various analogues.

---

1. Seeger, D. R., Cosulich, D. B., Smith, J. M., Jr., and Hultquist, M. E., *J. Am. Chem. Soc.*, 1949, v71, 1753.

2. Kidder, G. W., Dewey, V. C., and Parks, R. E., Jr., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 88.

3. Hutchison, D. J., and Burchenal, J. H., PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 251.

4. Burchenal, J. H., Waring, G. B., and Hutchison, D. J., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 311.

5. Zakrzewski, S. F., and Nichol, C. A., *J. Biol. Chem.*, in press.

6. Hutchison, D. J., and Burchenal, J. H., *Proc. Am. Assn. for Cancer Res.*, 1953, v1, 26.

7. Lepper, H. A., Editor: *Official Methods of Analysis of the Association of Official Agricultural Chemists*, Washington, D.C., 7th Edition, 1950, 784.

8. Sauberlich, H. E., *J. Biol. Chem.*, 1949, v181, 467.

9. Hutchison, D. J., and Burchenal, J. H., PROC. SOC. EXP. BIOL. AND MED., 1952, v80, 516.

10. Welch, A. D., and Nichol, C. A., *Ann. Rev. Biochem.*, 1952, v21, 633.

11. Goodall, R. R., and Levi, A. A., *Nature*, 1946, v158, 675.

12. Winsten, W. A., and Eigen, E., *J. Biol. Chem.*, 1950, v184, 155.

13. Hornibrook, J. W., *J. Lab. Clin. Med.*, 1950, v35, 788.

14. Nichol, C. A., *J. Biol. Chem.*, in press.

Received May 12, 1953. P.S.E.B.M., 1953, v83.

Encephalitogenic Properties of Crude and Protamine Treated Rabbit and Mouse Brain Suspensions. (20333)

PHILIP Y. PATERSON, WILLIAM L. POND, JOEL WARREN, AND MARVIN L. WEIL.

From the Department of Virus and Rickettsial Diseases and Department of Bacteriology, Army Medical Service Graduate School, Washington, D. C.

Man occasionally develops paralytic complications following the repeated injection of rabbit spinal cord during the course of the Pasteur treatment for rabies(1). Encephalomyelitis has been produced in several experimental animals following inoculation of homologous or heterologous brain tissue with(2-9) or without(10) the adjuvant mixture devised by Freund(11). These facts suggest that the vaccination of man with any preparation containing crude brain components may be hazardous.

The elimination of encephalitogenic activity from infected central nervous system tissue used for the preparation of several neurotropic virus vaccines would be of considerable merit. Such a method of preparing rabies vaccine has been reported by one group of workers(12,13). Previous reports from this laboratory(14,15) indicated that protamine treatment of infected crude brain material is useful for purifying a number of neurotropic viral agents. Furthermore, 2 mouse brain vaccines, Japanese and Russian Spring Summer encephalitis, prepared by this method gave initial immunologic responses comparable to crude preparations when tested in mice and human volunteers(16). Therefore, since this technic may have possible future application in the preparation of neurotropic viral vaccines of the mouse brain type, studies were carried out to determine the effect of protamine sulfate precipitation on the encephalitogenic activity of crude suspensions of rabbit and mouse brain tissue.

Materials and methods. Animals used for studies. All brain tissue was obtained from adult Swiss mice of the Bagg strain or adult, New Zealand White rabbits. The guinea pigs were bred at Walter Reed Army Medical Center and weighed 250 to 450 g each. The rhesus monkeys had been used several months previously for titrations of poliomyelitis virus and had shown no neurologic signs prior to use in these studies. *Preparation of brain tissue suspensions.* Freshly harvested mouse or rabbit brain tissue was prepared as a 20% suspension in buffered (pH 7.4) physiologic saline by use of a mortar and pestle or a Waring blendor. An aliquot of this preparation was used as the "crude uncentrifuged" brain suspension. A second aliquot was treated with powdered protamine sulfate (Lot No. 16693 or 1097397, Squibb & Co.) for 30 minutes at 4-6°C as previously described(14). A third untreated aliquot was held for a similar period of time at room temperature and subsequently centrifuged along with the protamine treated brain material in a mean gravitational field of 1200 g for 10 minutes. The resultant supernates were designated as the "crude supernate" and "protamine supernate" suspensions respectively. The sediments after resuspension in buffered saline to approximately their original volume served as the "crude sediment" and "protamine sediment" suspensions respectively. In 2 experiments designed to test the effects of freezing and thawing freshly harvested brain was stored at —20°C for 24 or 48 hours prior to being processed as described above. All brain-adjuvant emulsions were prepared in a mortar by mixing equal volumes of a given brain suspension and adjuvant mixture, the latter prepared according to the method of Freund(11). Each milliliter of final emulsion contained 0.5 ml brain suspension, 0.45 ml of liquid petrolatum, 0.05 ml of mannide mono-oleate (Arlacel-A, Atlas Powder Co.) and 2 mg of heat killed, dried *Mycobacterium tuberculosis*, strain H37Rv. *Inoculation of animals.* Each guinea pig received 3 subcutaneous inoculations of 1.5 ml of brain-adjuvant mixture along the dorsum at weekly intervals, except in Exp. 1 and 2, where only a single inoculum was administered. These animals were observed daily for neurologic signs for at least 4 and up to 8 weeks following the last inocula-

TABLE I. Incidence of Encephalomyelitis in Guinea Pigs Following Injection of Crude and Protamine Treated Rabbit and Mouse Brain Suspensions.

	Suspension injected				
	Crude			Protamine	
Brain tissue	Uncent.	Supe.	Sed.	Supe.	Sed.
Rabbit	10/15†	ND‡	ND	0/15	14/15
"	13/18	ND	ND	0/18	11/17
"	8/14	2/15	10/11	0/15	10/15
" *	11/13	5/12	7/12	0/12	8/12
" *	ND	6/15	ND	0/15	ND
Total incidence	42/60	13/42	17/23	0/75	43/59
Mouse	5/15	0/14	ND	ND	ND
"	8/12	1/12	6/12	0/12	4/12
"	6/12	2/12	6/12	0/12	4/12
Total incidence	19/39	3/38	12/24	0/24	8/24

* Frozen-thawed brain tissue employed.

† Numerator = No. of guinea pigs showing neurologic signs. Denominator = No. of guinea pigs inj.

‡ ND = Not done.

tion of brain material. The monkeys received 1.5 to 2.0 ml of a given brain-adjuvant suspension intramuscularly in each thigh on 2 occasions during a 3- to 6-week period and were observed daily for central nervous system signs for up to 3 months following the second inoculation.* *Criteria for evaluating encephalitogenic properties of inocula.* Encephalitogenic activity in a preparation was manifested by the development of paralysis and other neurologic signs in a group of inoculated guinea pigs. The absence of such clinical abnormalities in all animals of a group was taken as presumptive evidence that the suspension inoculated was devoid of encephalitogenic properties. In certain of these groups without neurologic signs histopathologic studies were carried out to detect subclinical encephalopathy. Brains and upper segments of spinal cord were fixed in 10% formalin, sectioned, and stained with hematoxylin and eosin for microscopic examination. Thus, the complete absence of both clinical signs and histopathologic lesions, as described by Lumsden(17), served to indicate that a given inoculum possessed little if any encephalitogenic activity. Microscopic study was not performed on the monkeys employed in this study and the sole criteria were the presence or absence of clinical neurologic signs.

Results. The results of 8 experiments in which 5 different types of rabbit or mouse brain suspensions were used to inoculate groups of guinea pigs are summarized in Table I. It will be noted that all crude brain preparations contained encephalitogenic activity although the actual incidence of neurologic signs varied greatly from experiment to experiment and from group to group. The highest incidence followed the inoculation of either crude uncentrifuged or crude sedimented material. Both these preparations produced signs in 50% or more of the animals in each group except in Exp. 6, where neurologic signs appeared in only 5 of 15 guinea pigs receiving crude mouse brain material. The crude suspensions following centrifugation, on the other hand, were less encephalitogenic and produced clinical signs of encephalopathy in less than 50% of every group. This decrease of encephalitogenic activity following centrifugation was most marked in Exp. 6, 7, and 8, where only 3 of a total of 38 guinea pigs developed neurologic signs after the inoculation of crude supernatant mouse brain material.

The effect of treating 7 of these crude suspensions with protamine sulfate will be noted in Table I. The protamine supernatant suspensions produced no paralysis or other neurologic signs in the 7 groups totaling 99 guinea pigs. Fifty-one of these animals, approximately half of which had received protamine supernatant material, prepared from rabbits and the remainder prepared from mice, were sacrificed for histopathologic studies. No microscopic encephalopathy was evident in any of these sections. Considerable encephalitogenic activity was demonstrable in the protamine sedimented inocula, all 6 of which elicited clinical signs when inoculated into animals.

The effect of protamine treatment was further demonstrated in an experiment employing 3 groups of monkeys consisting of 8 animals each. Crude uncentrifuged and protamine sedimented suspensions prepared from rabbit brain produced marked neurologic signs in 4 and 2 monkeys, respectively. None of the 8 monkeys in the third group which received protamine supernatant material developed clinical signs. It should be noted that, in an additional experiment, 10 monkeys received crude uncentrifuged mouse brain material and failed to develop clinical signs of encephalopathy during a 3-month observation period.

---

* The technical assistance of Dr. J. A. Morris in the monkey studies is gratefully acknowledged.

Discussion. The results of these studies indicate that protamine sulfate can precipitate most, if not all, of the encephalitogenic activity associated with crude rabbit and mouse brain tissue. The question might well be raised as to whether the single cycle of low speed centrifugation, used to sediment the protamine precipitated material, was responsible for this marked decrease in encephalitogenic activity. Centrifugation, as employed in these studies, did decrease the encephalitogenic character of the initial crude brain tissue. However, a small but significant amount of encephalitogenic activity remained in the majority of resulting supernatant fluids and thus, the effect of protamine treatment cannot be attributed to centrifugation *per se*.

These experiments were not designed to compare the difference in the encephalitogenic activity of crude rabbit and crude mouse brain tissue. However, it is worthy of comment that mouse brain, in general, appeared to be less encephalitogenic than rabbit brain tissue. This fact was suggested by the lower incidence of neurologic signs observed in guinea pigs receiving crude or centrifuged mouse brain suspensions and the failure of the former suspension to produce any abnormal signs in 10 monkeys. Neurotropic viral vaccines prepared from infected mouse brain tissue and clarified by low speed centrifugation have been employed for vaccination of man. Sabin has reported that this type of Japanese encephalitis vaccine resulted in no apparent paralytic complications when used during an outbreak of Japanese encephalitis on Okinawa during World War II(18). In this connection, it is of interest to mention that 2 vaccines prepared by the method of Sabin(19) from mouse brain infected with Japanese encephalitis virus were tested for encephalitogenic properties in this laboratory in 2 groups of 15 guinea pigs each. Neither vaccine, even when combined with adjuvants, produced any clinical signs of encephalopathy in this susceptible host. Such observations might be cause for minimizing the potential hazards associated with vaccination procedures employing this species of brain tissue. However, this study indicates that centrifugation alone cannot be relied upon to remove regularly encephalitogenic activity of both mouse and rabbit brain material. For this reason, the protamine technic may be of advantageous use in the preparation of certain neurotropic vaccines of the mouse brain type. Such vaccines would be expected to possess little, if any, encephalitogenic properties.

Conclusions. 1. Crude uncentrifuged rabbit or mouse brain tissue, combined with adjuvant, elicited acute encephalomyelitis in more than 50% of 99 guinea pigs. 2. The encephalitogenic activity of both types of brain tissue was decreased but not eliminated by low speed centrifugation. 3. Protamine sulfate appeared to sediment most, if not all, of the encephalitogenic activity of both rabbit and mouse brain material.

---

1. Sellers, T. F., *J. Med. Assn.*, Georgia, 1947, v36, 30.

2. Morrison, L. R., *Arch. Neur. and Psych.*, 1947, v58, 391.

3. Olitsky, P. K., and Yager, R. H., PROC. SOC. EXP. BIOL. AND MED., 1949, v70, 600.

4. Koprowski, H., and Jervis, G. A., PROC. SOC. EXP. BIOL. AND MED., 1948, v69, 472.

5. Kabat, E. A., Wolf, A., and Bezer, A. E., *J. Exp. Med.*, 1947, v85, 117.

6. Morgan, I. M., *J. Exp. Med.*, 1947, v85, 131.

7. Good, R. A., Campbell, B., and Good, T. A., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 341.

8. Thomas, L., Paterson, P. Y., and Smithwick, B., *J. Exp. Med.*, 1950, v92, 133.

9. Freund, J., Stern, E. R., and Pisani, T. M., *J. Immunol.*, 1947, v57, 179.

10. Rivers, T. M., and Schwentker, F. F., *J. Exp. Med.*, 1935, v61, 689.

11. Freund, J., and McDermott, K., PROC. SOC. EXP. BIOL. AND MED., 1942, v49, 548.

12. Hottle, G. A., Nedzel, G. A., Wright, J. T., and Bell, J. F., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 289.

13. Bell, J. F., Wright, J. T., and Habel, K., PROC. SOC. EXP. BIOL. AND MED., 1949, v70, 457.

14. Warren, J., *Bact. Rev.*, 1950, v14, 200.

15. Weil, M. L., Warren, J., Breese, S. S., Russ, S. B., and Jefferies, H., *J. Bact.*, 1952, v63, 99.
16. Warren, J., and Russ, S. B., unpublished data.
17. Lumsden, C. E., *Brain*, 1949, v72, 198.
18. Sabin, A. B., *J. Am. Med. Assn.*, 1947, v133, 281.
19. ———, PROC. SOC. EXP. BIOL. AND MED., 1947, v65, 127.

Received May 13, 1953. P.S.E.B.M., 1953, v83.

Effect of Toluidine Blue on Metastatic Calcification.* (20334)

ROGER BAKER, GERALD REAVEN, AND JOHN SAWYER. (Introduced by L. R. Dragstedt.)
From the Department of Surgery, Division of Urology, University of Chicago.

Hyperparathyroidism is the most common cause of metastatic calcification. Calcified lesions due to hyperparathyroidism have been reported in the connective tissue of the stomach, pulmonary alveoli and kidney(1). Within the kidney the peritubular basement membrane is involved. Gersh(2) considers the basement membrane as a plastic, well-organized ground substance that is highly labile in certain physiologic and pathologic states. Heller-Steinberg(3) and also Engel (4) have demonstrated that the histological changes seen in bone after administration of parathyroid extract, can be explained on the basis of depolymerization of connective tissue ground substance. Gersh and Catchpole(5) believe that calcification is most likely to occur in depolymerized matrix. Rubin and Howard(6) have confirmed this work and also observed that there is a relationship between the state of the connective tissue and the ability of the tissue to become calcified. They contend that more reactive groups are available to bind with calcium when the matrix is being depolymerized. The basic dye toluidine blue is known to combine with mucopolysaccharides of the ground substance. Sylven(7) and others believe this is due to the presence of chondroitin sulfate. Toluidine blue binds on reactive groups of the ground substance coinciding with calcified portions of the matrix. Furthermore, in three week-old rats in which calcification has not yet appeared, toluidine blue binds intensely with the matrix in precisely the geographical locations eventually to become calcified. Miller, *et al.*(8) have shown that *in vitro* calcification of rachitic rat cartilage is blocked by toluidine blue. These authors have demonstrated that this inhibition of calcification is directly proportional to the amount of toluidine blue present in the system. Marc and Wenk(9) and France(10) have reported that crystallization of certain inorganic salts from a supersaturated solution are markedly inhibited by dyes which stain the mother crystal.

Based on the above data, the object of this paper is to experimentally study the *in vivo* effect of dye binding on metastatic calcification produced in rats by the administration of parathyroid extract.

Methods. Young rats of the Sprague-Dawley strain, weighing 200-250 g and of the same sex, were used in the experiment. They were maintained on a routine laboratory diet and given water *ad libitum*. Fifteen rats each received a total dose of 1625 units of parathyroid extract (Lilly) administered over a 2 week period as described by Chown(11). Five rats each received 1625 units of parathyroid extract and 3 cc (intraperitoneally) of a 0.9 % saline solution each injection day of the experiment. Another group of 15 rats received the same dosage of parathyroid extract and in addition an intraperitoneal injection of 3 cc of a 0.04% solution of toluidine blue each injection day of the 2 week period. At the end of this time all animals were sacrificed by a blow to the head. Blocks of tissue obtained from the kidney, stomach, spleen, blood vessels and heart of each animal were fixed in absolute alcohol. Von Kossa and hematoxylin and eosin stains were obtained. To quantitate the extent of calcification in the

---

* This study was aided by Fred and Harvey Goldberg.

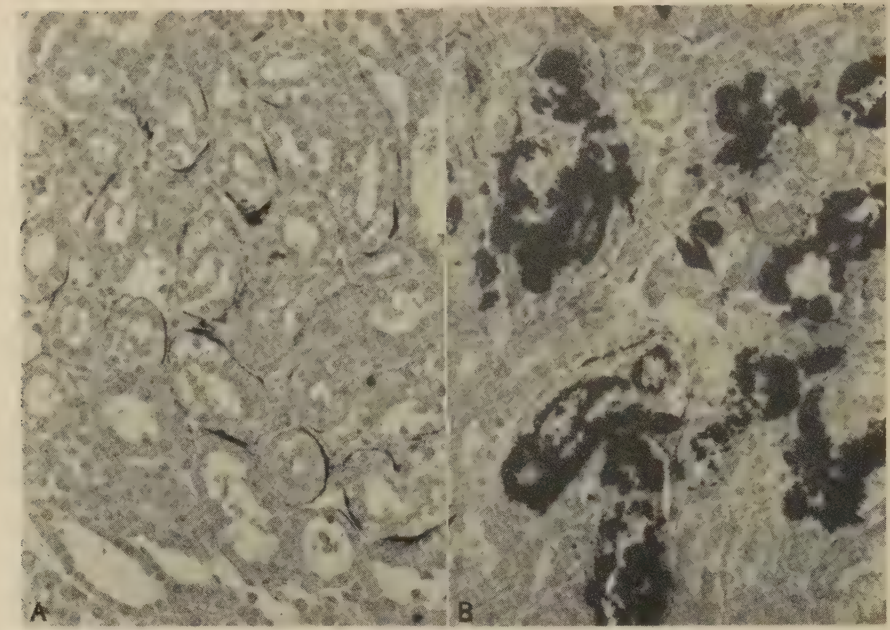


FIG. 1. A. Calcification in the basement membrane of the renal tubules in the rat produced by 1625 units of parathormone. (230 ×.)

FIG. 1. B. Augmentation of parathormone effect (1625 units) by concurrent administration of toluidine blue. (230 ×.)

kidney, four degrees of scoring were chosen. Those sections classified as 0 represent the stage at which no calcification, or at the most only small flecks of calcium, are seen in a few peritubular basement membranes. One plus refers to appreciable calcification, that is still localized peritubularly. When the calcium deposition appears in the basement membrane and also in the lumen of the tubules, it is termed 2 +; and 3 + refers to the situation in which the calcification is so extensive throughout the kidney that it is impossible to determine its exact location.

Results. Control animals generally demonstrated a uniform degree of metastatic calcification of the connective tissue ground substance in response to the 1625 units of parathyroid extract given. Calcification was observed in the kidney, blood vessels, spleen, heart and stomach. The administration of toluidine blue to animals receiving this same amount of parathormone greatly augmented metastatic calcification as compared to control animals given only parathormone. Figure 1 demonstrates the marked exaggeration of the parathyroid effect in the kidney produced by use of this basic dye. This augmentation of calcification was not confined to the kidney, but was observed also in the connective tissue matrix of the large and small arteries, spleen, heart and stomach (Fig. 2). The degree of calcification in the kidneys of the two groups of animals is presented in Table I. There was generally no calcification of the connective tissue ground substance in rats receiving a total two week dosage of 800 to 900 units of parathormone. The administration of toluidine blue in conjunction with 800 to 900 units parathormone, however, routinely resulted in calcification of 0 to 2 + in the ground substance of the organs and blood vessels previously described. Toluidine blue given alone failed to produce any calcification.

Summary and conclusions. 1. This experiment indicates that not only does toluidine blue fail to inhibit the metastatic calcification produced by parathormone, but that the deposition of calcium is augmented by the concurrent administration of the dye. Increased calcification was observed in the connective tissue ground substance of the kidney, stomach, spleen, arteries and heart. This was

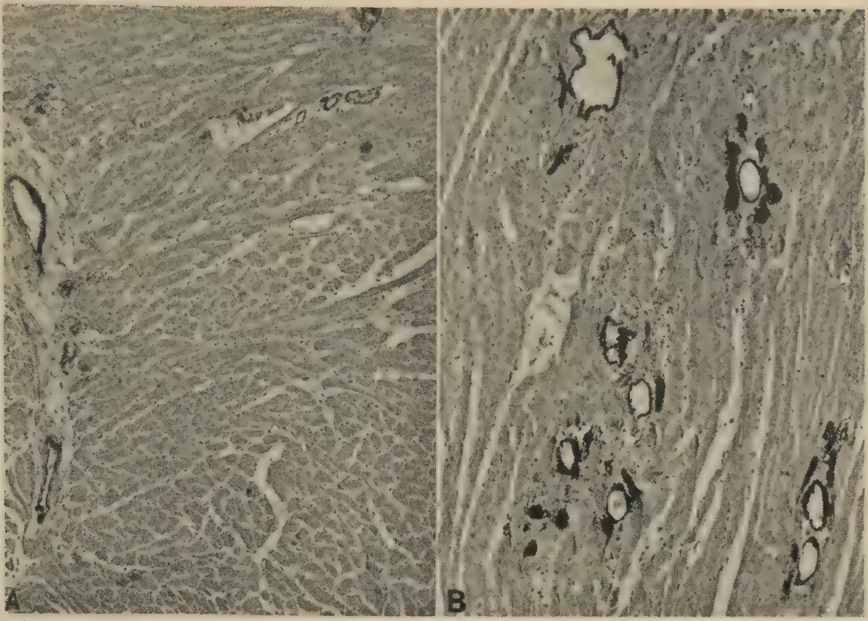


FIG. 2. A. Metastatic calcification of basement membrane of blood vessels produced by 1625 units of parathormone. (× 75.)

FIG. 2. B. Augmentation of parathormone effect (1625 units) by concurrent administration of toluidine blue. (× 75.)

an unexpected result due to the observation of Miller(8) that treatment of hypertrophic rachitic cartilage slices with basic dyes inhibits subsequent calcification. The fact remains, however, that the acid mucopolysaccharide present in the connective tissue matrix has a strong affinity for basic dyes. Rubin and Howard(6) have shown that the increased intensity of toluidine blue staining in areas about to calcify suggest that new polysaccharide appears or that the chemical state of the polysaccharide already present changes. An alteration in chemical form of the matrix favors combination with calcium and confers the state of calcifiability on the matrix. Engel (4) has reported that parathormone depolymerizes ground substance to increase calcifiability. It does not appear necessary therefore to infer competitive binding between toluidine blue and calcium for available reactive groups of the mucopolysaccharide. Concerning the data presented in this paper, toluidine blue apparently altered the chemical state of the ground substance, increased calcifiability, and exaggerated the parathormone effect. We have previously reported(12) that altering the connective tissue ground substance by compensatory renal hypertrophy or administration of desoxycorticosterone, increases or decreases, respectively, calcification produced by use of parathyroid extract. 2. The inhibitory effect of toluidine blue on the activity of heparin has been described by Allen(13). Experiments now in progress indicate that augmentation of calcification in the basement membrane of large and small arteries by use

TABLE I. Influence of Toluidine Blue on Metastatic Calcification.

No. of animals	Treatment	Duration, days	Degree of calcification in each exp. 0	+	++	+++
15	Parathormone (1625 units)	14	8	4	1	2
5	Parathormone (1625 units) and 0.9% saline	14	4	1	0	0
15	Parathormone (1625 units) and toluidine blue	14	0	0	5	10
8	Toluidine blue	14	8	0	0	0

of toluidine blue, as described in this paper, may be blocked by the concurrent administration of heparin.

1. Barr, D. P., *Physiol. Rev.*, 1932, v12, 593.
2. Gersh, I., *Harvey Lectures*, 1950, Series XLV, 211.
3. Heller-Steinberg, M., *Am. J. Anat.*, 1951, v89, 347.
4. Engel, M. B., *A.M.A. Arch. Path.*, 1951, v53, 369.
5. Gersh, I., and Catchpole, H. R., *Am. J. Anat.*, 1950, v85, 457.
6. Rubin, P. S., and Howard, J. E., *Trans. Macy Conf. on Met. Inter.*, 1950, v2, 155.
7. Sylven, B., *J. Bone and Joint Surg.*, 1947, v29, 745.
8. Miller, Z. B., Waldman, J., and McLean, F. C., *J. Exp. Med.*, 1952, v95, 497.
9. Marc, R., and Wenk, W., *Z. phys. Chem.*, 1909, v68, 104.
10. France, W. G., *Colloid Symp. Annual*, 1930, v7, 59.
11. Chown, B., Lee, M., Teal, I., and Currie, R. J., *J. Path. and Bact.*, 1939, v49, 273.
12. Baker, R., Reaven, G., and Sawyer, J., *J. Urol.*, in press.
13. Allen, J. G., Grossman, B. J., Elghammer, R. M., Moulder, P. V., McKeen, C. L., Jacobson, L. O., Pierce, M., Smith, T. R., and Crosbie, J. M., *J.A.M.A.*, 1949, v139, 1251.

Received May 14, 1953. P.S.E.B.M., 1953, v83.

Nutritional Requirements of *Lactobacillus bifidus* Isolated from Poults and Chicks.* (20335)

FRANCIS A. VELTRE, MARY S. SHORB, AND MICHAEL J. PELCZAR, JR.

(Introduced by R. D. Reid.)

From the Departments of Poultry Husbandry and Bacteriology, University of Maryland, College Park, Md.

Romoser *et al.*(1) reported that large numbers of an unidentified branched gram positive organism were found in ceca of normal chicks. These organisms, later identified as *Lactobacillus bifidus*(2), were found in greatest numbers when 15% lactose was added to the basal diet and were decreased in number by the addition of 10 ppm of penicillin G. A high bifid count was correlated with poor chick growth. *L. bifidus* also constitutes 50% of the cecal population of turkeys(3). It is well known that under the usual growing conditions, poults grow faster when antibiotics are fed. This may be due to a reduction in numbers of bifid organisms. Since *L. bifidus* might utilize a known or unidentified substance(s) essential for rapid chick and poult growth, a knowledge of the nutritional requirements of avian bifids may contribute information concerning the mechanism of antibiotic action(4-6). Results of such a study are presented in this paper.

Experimental. Twenty-four cultures, tentatively classified as *L. bifidus*, have been isolated from the ceca of turkey poults. For the nutritional studies, 7 representative avian strains were selected, including 5 poult strains, one strain from adult turkeys(3) and one strain from chicks(2). The general procedures used in microbiological assays were employed for the determination of growth requirements. Stock cultures were transferred monthly on the basal medium containing 15 g per liter of Phytone (papaic digest of soybean, Baltimore Biological Laboratory) and 2% agar. The medium used for preparation of inoculum was of the same composition except for the omission of agar. Stock and inoculum cultures were incubated at 37°C, for 48 hours under anaerobic conditions using a Brewer anaerobic jar. The inoculum cultures were centrifuged, washed twice with physiological saline, resuspended and adjusted to a reading of 95 on the Evelyn photo-electric colorimeter, using

---

* Scientific Paper No. A 418. Contribution No. 2450 of the Maryland Agric. Exper. Station.

This investigation was supported in part by a contract between the Office of Naval Research, Department of Navy, Washington, D.C. and the University of Maryland.

a 660 mμ filter. One drop of this suspension was added to each of the assay tubes. Solutions were prepared so that the amount of supplement for each test was contained in 1 to 3 ml. In all cases the final volume in each tube was 6 ml. The basal medium was identical to that employed by Hassinen *et al.*(7) except for the following modifications: the lactose and sodium acetate were reduced to 7 and 5 g per liter of single strength medium, respectively. Cystine was replaced by cysteine hydrochloride and N-Z Case (enzymatic digest of casein) by Vitamin-Free Casamino Acids (Difco) or amino acid mix(8). Alanine and tryptophane were omitted when amino acid mix or Casamino Acids were used as the nitrogen source. Ascorbic acid was omitted except where indicated in the results. The final pH of the medium was adjusted to 6.8. The tubes were autoclaved at 15 lb pressure for 15 minutes, cooled, inoculated and incubated anaerobically at 37°C for 48 hours. The growth was measured turbidimetrically in the Evelyn colorimeter, using the 660 mμ filter.

Results and discussion. None of the 7 strains would grow in several partially chemically defined media, including the medium described by Hassinen *et al.*(7). Preliminary investigations using Eugon broth revealed that Phytone was necessary for growth; however, upon the addition of this supplement to Hassinen's medium, no growth was obtained. Thus, it was surmised that the concentration of ingredients was the factor responsible for the absence of bacterial growth. Under our experimental conditions, the avian bifids were inhibited by the concentration (25 mg per ml) of sodium acetate used by Hassinen *et al.*(7), Tomarelli *et al.*(9) and Kuhn(10) for the proliferation of *L. bifidus* of human origin. A low concentration (5 mg per ml) improved growth of avian bifids. The reduction of lactose from 35 to 7 mg per ml did not appreciably decrease growth. Cystine and cysteine hydrochloride were interchangeable, but cysteine hydrochloride was chosen for incorporation in the basal medium because of its non-toxic effect(11) and its reducing properties.

TABLE I. Effect of Phytone and Ascorbic Acid on Growth of *Lactobacillus bifidus*. (Poult Strain 3.)

	Optical density*			
	No ascorbic acid		Ascorbic acid†	
Phytone conc., mg/tube	Aerobic	Anaerobic	Aerobic	Anaerobic
0 (Inoc.)	.00	.00	.00	.00
5	.04	.07	.13	.09
10	.09	.11	.14	.11
20	.13	.21	.21	.21
40	.12	.39	.40	.44
60	.14	.48	.62	.51
90	.32	.64	.75	.70

* $\log \frac{100}{\text{Galvanometer reading}}$.

† 12 mg/tube.

The 7 avian strains, when grown in the modified basal medium described above, had an absolute requirement for a growth-promoting substance(s) in Phytone. This growth response is shown in Table I. The substance was also present in yeast extract, beef extract and fish solubles. The latter crude material is well known to contain growth-promoting substances for chicks and turkeys.

None of the following compounds singly or in combination replaced the Phytone requirement: vit. B_{12}, thymidine, desoxyribonucleic acid, choline, inositol, lyxoflavin, carnitine, Leucovorin (citrovorum factor), DL-6-thioctic acid (protogen), pantethine, pyridoxal hydrochloride, pyridoxal phosphate, pyridoxamine, orotic acid, oleic acid, polyoxyethylene sorbitan monooleate (Tween 80), blood group specific substances A and B, and galactose acetylglucosamine. It has been reported that the growth factor in human milk(12) required by variants of human bifids, could be replaced by galactose acetyl-glucosamine(13) or a nitrogen-containing polysaccharide from mucin(10) or partially replaced by blood group specific substances A and B(10). The variant human strains have not been made available for comparison in our tests. However, the unidentified factor required by the avian bifids appears to be different from that required by the variants of human bifids since galactose acetyl-glucosamine and blood group specific substances A and B failed to replace the Phytone activity when incorporated in the medium described by the above authors or when incorporated in the basal medium used in our experiments.

The avian bifids also seem to be different from the human bifids, in that they exhibit a marked stability in oxygen requirement and bifid morphology. In contrast to the behavior of a number of bifids isolated from nursling stools of infants(9,14), they have not become aerobic nor lost their branched form upon continual subculture even after a period of one to 2 years. However, they can be grown in an aerobic atmosphere, provided ascorbic acid is added either aseptically or autoclaved in the medium.

In Table I the effect of ascorbic acid on the growth of one culture (poult strain No. 3) is shown. That some aerobic growth was obtained with high levels of Phytone in the absence of ascorbic acid was not too surprising, since a crude substance such as Phytone might contain a reducing compound. There was no apparent change in bifid morphology when the organism was grown in an aerobic atmosphere in the presence of ascorbic acid. From the results obtained, the bifids isolated from ceca of chicks and poults appear to be more fastidious in their nutritional requirements than the bifids from human origin.

Summary. The nutritional requirements of *Lactobacillus bifidus* isolated from the ceca of chicks and turkey poults have been studied. All strains were inhibited by high concentrations of sodium acetate. The organisms could be grown in an aerobic atmosphere, provided ascorbic acid was present, without any apparent change in bifid morphology. No growth was observed when a chemically defined medium was employed, but luxuriant growth was obtained in the basal medium when it was supplemented with Phytone or other crude substances. The activity of Phytone could not be replaced by the galactose acetyl-glucosamine or the incorporation of several other known growth-promoting substances into the basal medium. The nutritional fastidiousness of *L. bifidus* isolated from chicks and poults may suggest that these organisms compete with the host for mutually essential nutrients.

The authors are indebted to Merck and Co., Inc., Rahway, N. J., for providing vit. B_{12}, lyxoflavin, pyridoxal phosphate, and pantethine; to Lederle Laboratories, Inc., Pearl River, N. Y., for DL-6-thioctic acid and Leucovorin; to Dr. Edmond Snell, University of Texas, Austin, Texas, for thymidine; to Dr. Herbert Carter, University of Illinois, Urbana, for carnitine; to Sharp and Dohme, Philadelphia, Pa., for biocytin and blood group specific substances A and B; and to Wyeth, Inc., Mason, Mich., for galactose acetyl-glucosamine.

---

1. Romoser, G. L., Shorb, M. S., Combs, G. F., and Pelczar, M. J., Jr., *Antibiotics and Chemotherapy*, 1952, v11, 42.

2. McCarthy, R. E., Masters Thesis, 1953, University of Maryland.

3. Harrison, A. P., Jr., and Hansen, P. A., *J. Bact.*, 1949, v59, 197.

4. Romoser, G. L., Shorb, M. S., Combs, G. F., *Poultry Sci.*, 1952, v31, 932.

5. Anderson, G. W., Slinger, S. J., and Pepper, W. F., *Poultry Sci.*, 1952, v31, 905.

6. Cook, F. D., Blankeley, R. M., MacGregor, H. I., and Anderson, R. W., *Poultry Sci.*, 1952, v31, 912.

7. Hassinen, J. B., Durbin, G. T., Tomarelli, R. M., and Bernhart, F. W., *J. Bact.*, 1951, v62, 771.

8. Shorb, M. S., PROC. SOC. EXP. BIOL. AND MED., 1952, v79, 611.

9. Tomarelli, R. M., Norris, R. F., and György, P., *J. Biol. Chem.*, 1949, v181, 879.

10. Kuhn, R., *Angewandte Chemie*, 1952, v64, 495.

11. Rose, C. S., Feldbaum, E. P., Norris, R. F., and György, P., PROC. SOC. EXP. BIOL. AND MED, 1953, v81, 709.

12. György, P., Kuhn, R., Norris, R. F., Rose, C. S., and Zilliken, F., *Amer. J. Dis. Child.*, 1952, v84, 482.

13. Tomarelli, R. M., Linden, E., and Bernhart, F. W., *Fed. Proc.*, 1953, v12, 431.

14. Weiss, J. E., and Rettger, L. F., *J. Bact.*, 1934, v28, 501.

Received May 14, 1953. P.S.E.B.M., 1953, v83.

Half-Lives of Homologous Serum Albumins in Several Species.* (20336)

Frank J. Dixon, Paul H. Maurer, and Maria P. Deichmiller.

From the Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, Pa.†

It has been shown that the half-lives of homologous gamma globulins vary from species to species(1). As a corollary, the half-lives of homologous serum albumins have been determined using the I^{131} protein label, a convenient tool for the measurement of protein catabolism.

Materials and methods. Albumin was separated from human, bovine, and rabbit serum in the laboratories of Armour and Co. according to the alcohol fractionation procedure of Cohn(2). Albumin from dog and mouse serum was obtained by electrophoretic separation in a Tiselius apparatus in the laboratory of Dr. D. H. Moore of Columbia University. To the albumins in a pH seven 0.2 M phosphate buffer was added a slightly acid solution of I^{131} as free iodine and then the pH of the mixture was raised to 9 by the addition of 0.1 N NaOH. Non-protein-bound iodine was eliminated by dialysis against refrigerated tap water for 72 hours. The entire procedure was carried out at 0°-5° C. Final preparations contained less than one iodine atom per molecule of protein and at least 99% of the radioactivity was protein bound, as determined by trichloracetic acid precipitation. (1) The following subjects were used: Eleven men ages 23 to 75 who were institutionalized in a county home. These men had the following diagnoses: 5 - multiple sclerosis, 2 - hemiplegia old, one - healed osteomyelitis, one - chronic arthritis, one - post operative bladder cancer, and one - asthma. (2) Three nonlactating full grown Swiss and Jersey cows. (3) Nine full grown mongrel dogs. (4) Nine young albino male rabbits weighing between 2 and 2.2 kg for rabbit albumin half-life determination. (5) Eight young albino male rabbits weighing between 2 and 2.2 kg for the determination of the effect of metabolic rate on albumin half-life. (6) Thirty young adult CFW strain female mice weighing from 20 - 21 g. All subjects were given oral potassium iodide supplements to saturate iodine utilizing tissues and minimize retention of I^{131} liberated by catabolism of labelled albumin. I^{131} labelled homologous albumin, in amounts less than 10 mg per kilo body weight, was injected intravenously into all species except the mouse in which injections were made subcutaneously. Two or more days were allowed after injection for the labelled protein to equilibrate completely between the intravascular and extravascular components of the plasma protein pool before the half-life determinations were started. After equilibration the humans, cows, dogs and rabbits were bled at six or seven day intervals and the concentration of protein bound I^{131} in the plasma was determined(3,4). All 30 mice received identical injections of labelled albumin and ten were exsanguinated 3, 5, and 7 days later. The blood from the mice in each group was pooled and labelled protein concentration of the pooled plasma was determined.

As was suggested by our earlier study of gamma globulin half-life(1) the metabolic rate of the host appeared to influence the protein half-life. To further test this hypothesis we determined the albumin half-life in rabbits made hypermetabolic by daily injections of 0.1 to 0.2 mg of thyroxin per kilo body weight throughout the period of observation.

Results and discussion. The half-lives of I^{131} labelled homologous albumins were: cows, 20.7 days; humans, 15.0 days; dogs, 8.2 days; rabbits, 5.7 days; and mice, 1.2 days (Table I). These values are calculated for the entire period of observation indicated on Table I. In spite of obvious differences in structure, size, origin, and function of albumin and gamma globulin, the albumin half-lives correspond closely to the gamma globulin half-lives reported earlier: cow, 21.2 days; human, 13.1 days; dog, 8.0 days; rabbit, 4.6-5.7 days; and mouse, 1.9 days(1). It would appear that

---

* This study was supported by Atomic Energy Commission contract.

† This is No. 23 of the Pathology Department, University of Pittsburgh School of Medicine.

TABLE I. Albumin Half-Lives.

Species	No. of subjects	Method of fractionation	Interval after inj. for which half-life is calculated in days	Half-life with stand. dev. in days
Cow	3	Alcohol	2–30	20.7 ± 1.1
Human	11	”	7–28	15.0 ± 1.9
Dogs	9	Electrophoresis	7–28	8.2 ± 1.2
Rabbit	9	Alcohol	4–22	5.7 ± .3
Mouse	30	Electrophoresis	3– 7	1.2

the dominant role in the catabolism of these proteins is played by the metabolic characteristics of the host and not by the characteristics of the protein molecules themselves.

The same inverse relationship between protein half-life and metabolic rate of the host noted previously for gamma globulin also held for albumin. This relationship was further supported by the finding of a 4.4 ± 0.3 day albumin half-life in the 8 rabbits given thyroxin. The metabolic rates of these animals were increased an average of 120% as indicated by measurement of oxygen consumption with a Benedict Roth metabolor attached to an airtight cage.

The homologous albumin half-lives reported here agree fairly well with those reported by others. Using the same technique Sterling found an albumin half-life of 10.5 ± 1.5 days in medical students(5). The difference between this figure and the 15 day value we found for institutionalized males may be largely the result of the difference in activities and, therefore, metabolic rates of the two groups of subjects. Tracing albumin labelled with N^{15}, London found a half-life of approximately 20 days in humans(6). This value would be expected to be somewhat greater than the true half-life since some of the N^{15} label is used in resynthesis of protein after catabolism of the originally labelled protein. Calculations based on the 2 to 6 day post injection determinations of Fink, *et al.*, give an 8.4 day half-life for total plasma protein in dogs(7) which agrees well with our 8.3 day albumin half-life and 8.0 day gamma globulin half-life.

Summary. The half-lives of homologous serum albumins in humans, cows, dogs, rabbits and mice have been presented. The albumin half-lives are similar to the gamma globulin half-lives for the same species and apparently are dependent upon the metabolic characteristics of the host.

This study has been greatly facilitated by the cooperation of the Allegheny County Institution District, Mayview Hospital, Mayview, Pa.; the Addison Gibson Laboratories of the University of Pittsburgh School of Medicine; and Dr. H. Salk, Mars, Pa.

---

1. Dixon, F. J., Talmage, D. W., Mauer, P. H., and Deichmiller, M. P., *J. Exp. Med.*, 1952, v96, 313.

2. Cohn, B. J., Strong, L. E., Hughes, W. L., Jr., Mulford, D. J., Ashworth, J. N., Melin, M., and Taylor, H. L., *J. Am. Chem. Soc.*, 1946, v68, 459.

3. Dixon, F. J., Bukantz, S. C., and Dammin, G. J., *Science*, 1951, v113, 274.

4. Talmage, D. W., Dixon, F. J., Bukantz, S. C., and Dammin, G. J., *J. Immunol.*, 1951, v67, 243.

5. Sterling, Kenneth, *J. Clin. Invest.*, 1951, v30, 1228.

6. London, I. M., *Plasma Proteins*, (John B. Youmans, editor), Robert Gould Research Foundation, Springfield, Ill., 1950, 72.

7. Fink, R. M., Enns, T., Kimball, C. P., Silbestein, H. E., Bale, W. F., Madden, S. C., Whipple, G. H., *J. Exp. Med.*, 1944, v80, 455.

Received May 15, 1953. P.S.E.B.M., 1953, v83.

Role of Host Defenses in Streptomycin Treatment of X-Irradiated Mice.* (20337)

ROBERT Q. MARSTON,† H. JEANETTE RUTH, AND WILLIE W. SMITH.
From the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health, Public Health Service, Department of Health, Education, and Welfare, Bethesda, Md.

The complementing action of natural defenses in the control of various infections with sulfonamides and antibiotics has been demonstrated in several ways. Wood and his colleagues(1) showed the importance of surface phagocytosis in sulfonamide treatment of pneumonia; Skinsness and Woolridge(2) found the effectiveness of penicillin against pneumococcal infections to be reduced in protein-depleted rats; and Kaplan and his associates(3) showed aureomycin to be relatively less effective against β hemolytic streptococcal infection in irradiated than in non-irradiated mice.

In view of these and related findings one may predict that the effectiveness of prophylactic antibiotic treatment after whole-body X-irradiation will depend to a considerable degree upon the residual level of the severely injured host defenses. The probability that such is the case is apparent in our results with streptomycin treatment of natural and experimental infections in irradiated mice(4-6), where deficiencies in streptomycin effectiveness were not adequately explained by the drug-resistance of invading or injected organisms or by inadequacies in dose or duration of treatment. Jacobson(7) and Lorenz(8) demonstrated the increased hematopoietic recovery and increased survival which results from spleen transplants or bone marrow injection after irradiation. Cole and his associates(9) obtained similar results with the administration of spleen homogenate from immature mice. Our group has demonstrated a close correlation between the degree of leucocyte recovery, with and without the injection of spleen homogenate, and resistance to experimental infections(10). Results with streptomycin treatment of experimental infection in irradiated mice whose recovery is accelerated by the administration of spleen homogenate are reported here.

Procedures. Male mice of the N.I.H. strain were caged individually and distributed by litter into treatment and irradiation groups as previously described(4). Each irradiated infected control group contained 20 mice and each of the other groups 36 to 40 mice. At 12 weeks of age they were given 475r (LD about 5%) with an X-ray unit operated at 200 KV and 20 ma with 0.25 mm Cu and 0.51 mm Al added filtration. The target distance was 50 cm. Spleen homogenates were prepared by a method similar to that of Cole *et al.*(9). Spleens from decapitated 2- to 3-week-old mice were removed, freed from adhering adipose tissue, placed in Earle's buffer(11) and ground, in batches of about 30, in a Potter-Elvehjem type homogenizer. More buffer was added to give a final dilution equivalent to 2 spleens per ml. The suspension was allowed to stand a few minutes until large clots appeared and was then filtered twice through thin layers of glass wool. Within a few hours after irradiation each mouse was given a single injection of 0.5 ml into a tail vein through a No. 24 needle. Mice from each treatment group were given the injections in rotation to minimize variations in spleen preparation and in time of injection. *Proteus Kf7* (Kaufmann), an organism foreign to our mice, was used for infection. A lyophilized culture was inoculated into horse meat infusion broth and incubated at 37°C for 24 hours. A 24-hour subculture was centrifuged and the organism resuspended in sterile saline to give a 1:10 dilution of the subculture for immediate injection. Each mouse was given 0.2 ml subcutaneously in the region of the groin. Injections of 1.25 mg streptomycin sulfate in 0.2 ml were given under the dorsal skin daily beginning 5 hours after the injection of *Proteus*. This dose was

---

* Supported in part by a grant from The Department of Defense.

† M. C., U. S. Army.

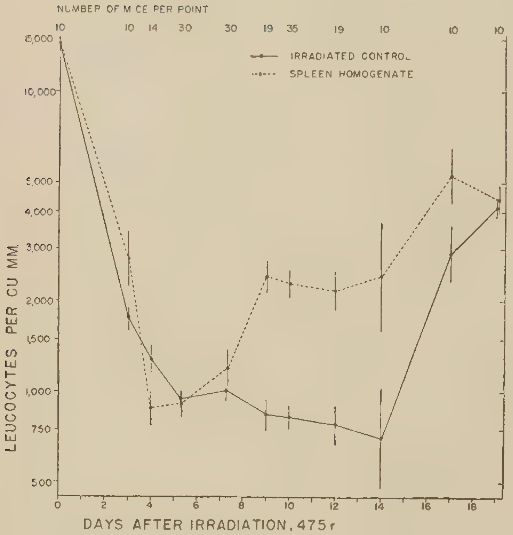


FIG. 1. Geometric means and stand. errors of leucocyte counts with and without inj. of spleen homogenate within a few hr after irradiation.

half of that previously found to be approximately optimal for the treatment of experimental *Proteus* infection(6). Leucocyte counts were made from tail blood. The values are shown as geometric means with standard errors and are derived from 5 experiments in which irradiation and the injection of spleen homogenate were essentially the same as in the experiment reported in detail here.

Results. A composite graph showing the effect of spleen homogenate on leucocyte count is given in Fig. 1. The start of recovery is usually apparent by the 7th day, while in the irradiated controls the counts usually remain below 1000 per cu mm for 14 days.

Survival curves following inoculation with *Proteus* are shown in Fig. 2. The 4 groups in the first block had been irradiated 5 days previously and parallel groups in the second and third blocks 7 and 10 days prior to infection. Most of the deaths occurred in less than 2 days after infection. Of 19 non-irradiated mice 2 died following the *Proteus* inoculation. In each untreated irradiated group 90 to 100% died after infection. In the spleen-treated group that was infected on the 5th day there was little improvement in survival, but with infection on the 7th or 10th day almost half of the mice survived. Similarly, results with streptomycin were better in mice infected 7 or 10 days after irradiation.

Although the injection of spleen homogenate caused no perceptible increase either in leucocyte count or in resistance to infection by the 5th day after irradiation, it had, at that time, caused considerable improvement in the efficacy of streptomycin treatment, increasing survival from 8% to 47%. The leucocyte count on the 7th day was not much higher in spleen-treated than in non-treated mice, but it was increasing rapidly and the efficacy of streptomycin in the spleen-treated group at that time, as well as 3 days later, was improved from a level of about 45% to approximately 75%.

Discussion. From the studies carried out in Miller's laboratory(12) and in our own (4,5) it is clear that, in mice, infection is a determining factor in postirradiation death or survival. Further experiments(10) strongly suggest that in this species the efficacy of postirradiation injection with spleen homogenate is largely dependent upon its action in restoring defenses against bacterial invasion. Although such treatment accelerates recovery of all hemopoietic elements, phagocytosis stands out as the logical factor of critical importance in the experimental conditions employed here. By giving sub-lethal irradiation and injecting the organism subcutaneously, intestinal epithelial barriers were by-passed; when infection was given on day 5 or 7 the experiment was essentially complete before anemia developed; and by injecting an organism foreign to our mice which kills in less than 48 hours, the possible contribution of specific antibodies was almost certainly excluded. The extent to which preformed nonspecific antibodies may influence resistance to infection under the conditions of these experiments is not known.

The results here demonstrate a dependence of streptomycin therapy upon the level of host defenses, both in natural recovery and in recovery under the influence of injected spleen homogenate. The extent of this dependence is particularly critical for the irradiated mouse during the first 2 weeks when the leucocyte count is very low and antibody formation has ceased. This depleted state of the host defenses may reasonably account for many of the undramatic results frequently observed

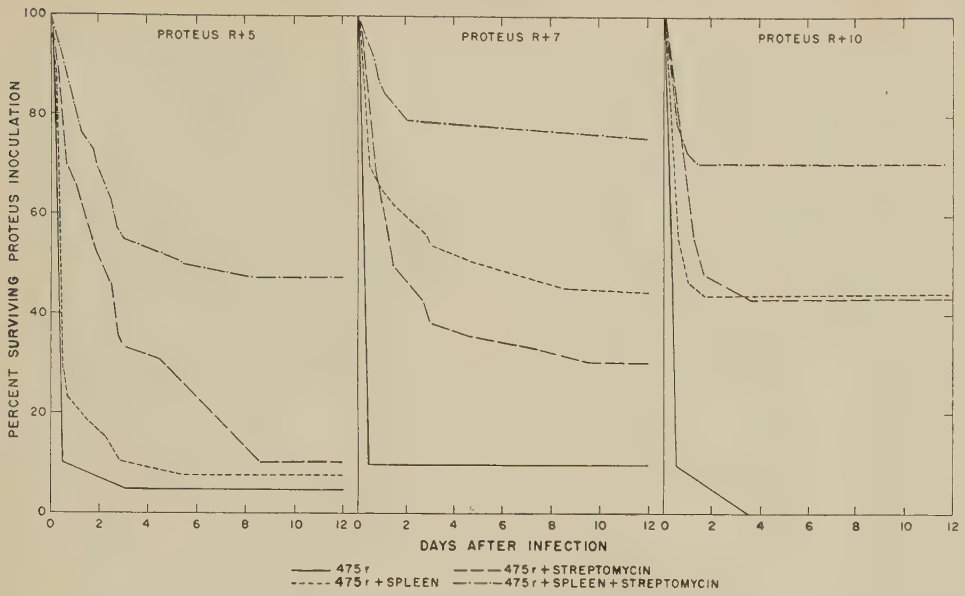


FIG. 2. Cumulative survival curves following subcutaneous inj. of *Proteus Kf7* 5, 7, and 10 days after irradiation.

with antibiotic therapy after whole-body X-irradiation(4,12).

Summary. The intravenous injection of a homogenate of immature spleen increases the resistance of sub-lethally irradiated mice to *Proteus* infection and significantly complements the action of streptomycin.

---

1. Wood, B. W., Jr., Smith, M. R., and Watson, B., *J. Exp. Med.*, 1946, v84, 387.

2. Skinsness, O. K., and Woolridge, R. L., *J. Infect. Dis.*, 1948, v83, 78.

3. Kaplan, H. S., Speck, R. S., and Jawetz, E., *J. Lab. Clin. Med.*, 1952, v40, 682.

4. Smith, W. W., Smith, F., Ruth, H. J., Canter, H. Y., and Grenan, M. M., *Am. J. Physiol.*, 1953, v172, 351.

5. Gonshery, L., Marston, R. Q., and Smith, W. W., *Am. J. Physiol.*, 1953, v172, 359.

6. Marston, R. Q., Gonshery, L., Alderman, I. M., and Smith, W. W., *Am. J. Physiol.*, 1953, v172, 365.

7. Jacobson, L. O., Simmons, E. L., Marks, E. K., Gaston, E. O., Robson, M. J., and Eldredge, J. H., *J. Lab. Clin. Med.*, 1951, v37, 683.

8. Lorenz, E., Congdon, C., and Uphoff, D., *Radiology*, 1952, v58, 863.

9. Cole, L. J., Fishler, M. C., Ellis, M. E., and Bond, V. P., PROC. SOC. EXP. BIOL. AND MED., 1952, v80, 112.

10. Smith, W. W., and Marston, R. Q., *Fed. Proc.*, 1953, v12, 135.

11. Earle, W. R., *J. Nat. Cancer Inst.*, 1943, v4, 165.

12. Miller, C. P., Hammond, C. W., and Tompkins, M., *J. Lab. Clin. Med.*, 1951, v38, 331.

Received May 18, 1953. P.S.E.B.M., 1953, v83.

Use of Vi Phage Lysates in Genetic Transfer. (20338)

L. S. Baron, S. B. Formal, and W. Spilman. (Introduced by M. Landy.)

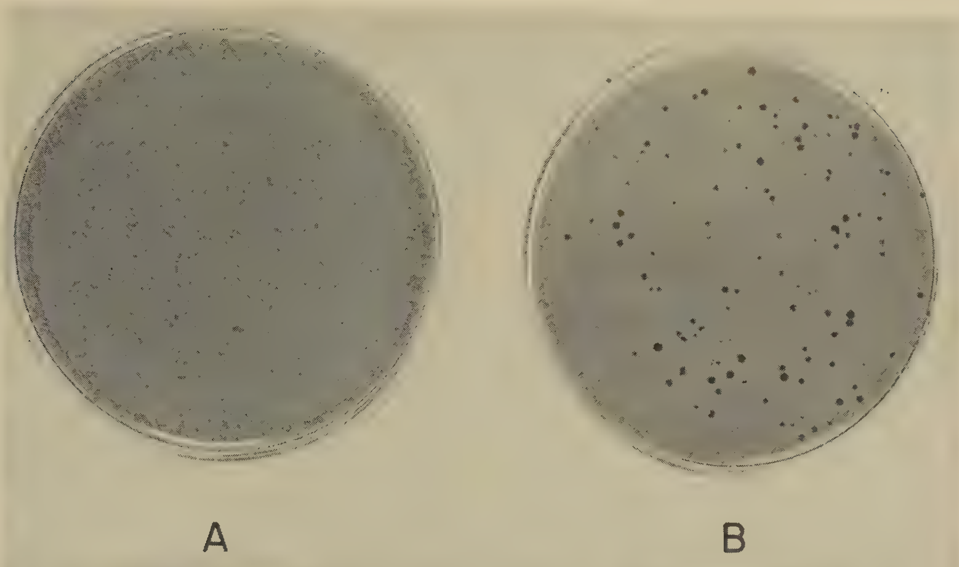
From the Immunology Division, Army Medical Service Graduate School, Walter Reed Army Medical Center, Washington, D.C.

Recent investigations by Zinder and Lederberg(1) have indicated that genetic exchange in Salmonella is mediated by a bacterial product which they have called FA (filterable agent). While the active filtrates utilized in these experiments possess the ability to transfer (transduce) many hereditary traits from one strain to another, indications are that for the most part factors are transferred singly by this mechanism. It has also become evident that the effectiveness of the initial filtrates depends upon the presence and activation of lysogenic phage. There seems, in addition, to be good reason to suspect these phage particles as the actual transducing agents, especially if one considers the interpretation of variation in bacterial viruses proposed by Bertani and Weigle(2). These workers have assumed the existence of a phage structure the specificity of which is completely or almost completely under control of the host cell.

Preliminary investigations in this laboratory have revealed an analogous system for genetic transfer which makes use of Vi phage lysates, thereby eliminating the necessity for induction of lysogenicity as a prerequisite in the preparation of active filtrates.

Materials and methods. Lysates are produced by the action of specific Vi phage on strains of *Salmonella typhosa* employing the usual methods described by Adams(3). For these experiments, sterile stocks of Vi phage E_1 were prepared by lysis of the donor strain, a streptomycin resistant mutant of *S. typhosa* strain Ty2, which is xylose positive and antigenically typical. The serotype of this culture is represented as Vi, IX, XII:d. Genotypically, the factors under study may be depicted as follows: Xyl+, S^r, designating the ability to ferment xylose and resistance to streptomycin. The number of phage particles present in the lysate can be accurately determined by the plaque count method and in general, prepared stocks are usually found to contain 10^9 to 10^{10} phage particles per ml. Receptor strains consist of a number of Vi cultures of *S. typhosa* which are able to absorb the phage from the lysate without the occurrence of lysis. Assays of lysate supernates have indicated that more than 90% of phage particles originally present are absorbed by receptor strains. The basic experimental procedure involves the incubation of receptor cells in the presence of a phage lysate prepared from the donor strain. As controls, equal portions of the original suspension are suspended in both nutrient broth and boiled filtrate. After a 30 minute incubation period, the suspensions are washed, taken up in a few ml of saline and assayed for cell count. These suspensions are then examined for the presence of xylose positive and streptomycin resistant cells.

Results. *S. typhosa* strain 643 possesses an antigenic structure comparable to the donor strain, but is xylose negative and streptomycin sensitive (Xyl–, S^s). Cell suspensions of this strain after treatment with E_1 phage lysates of the donor culture are plated on Eosine-Methylene Blue (EMB) xylose plates to select cells transduced for the ability to ferment xylose. Approximately 10^9 cells spread on EMB xylose plates have given rise to varying numbers of xylose positive cells depending upon the number of phage particles present in the original lysate. No positives have ever been observed on the control plates although uninhibited negatives appear in comparatively small number on both control and experimental plates (Fig. 1). The great majority of cells of strain 643 are completely inhibited when plated on EMB xylose plates. As a further check on spontaneous mutability of the xylose factor, fermentation tubes containing xylose broth with phenol red as an indicator were inoculated with control suspensions. Tubes were held at least 30 days and despite the use of dense suspensions of untreated cells no positive tubes were observed in over 200 fermentation tests. On the other hand, all tubes inoculated with phage treated



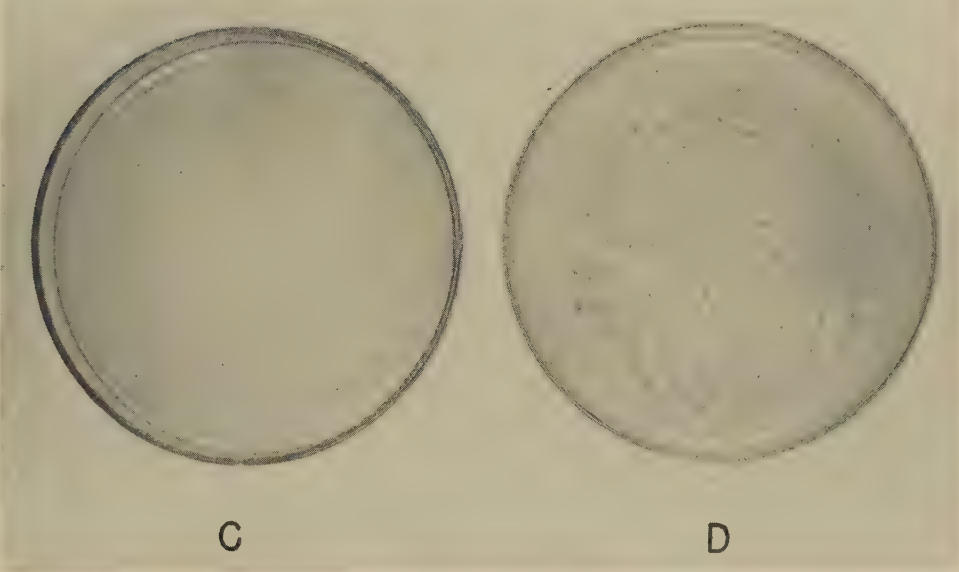


FIG. 1 (top). *Transfer of xylose fermenting ability.* Demonstrated by plating (A) cells treated with heat inactivated phage lysates, (B) cells treated with active phage lysates, on EMB xylose plates.

FIG. 2 (bottom). *Transfer of streptomycin resistance.* (C) cells treated with heat inactivated phage lysates, (D) cells treated with active phage lysates.

cells of the normally xylose negative strain 643 became positive in 2-3 days. Diluted suspensions of treated cells calculated to contain about 10 to 20 transduced positives fermented xylose in a majority of the tubes. All tubes were further checked by streaking on EMB xylose plates. In addition, large numbers of positive clones from EMB xylose plates were tested by streaking on nutrient agar plates containing 0.5 mg of streptomycin per ml and were uniformly found to remain streptomycin sensitive.

Experiments were undertaken designed to demonstrate a transfer of streptomycin resistance from the donor culture, using phage lysates, to strain 643 as well as to a serologi-

TABLE I. Transfer of Streptomycin Resistance to *S. typhosa* Strain 643.
Streptomycin resistant clones.

Control cells	Phage treated cells
0	110
0	160
0	108
1	98
0	108
1	115
0	99

10^9 cells spread on nutrient agar plates and incubated 2 hr at 37°C. After incubation, plates were overlaid with .7% agar containing .5 mg streptomycin/ml.

cally rough culture, *S. typhosa* strain Vi I (Bhatnagar) which is Vi positive, non-motile and essentially devoid of the typhoid somatic antigens. Experimental and control suspensions prepared as previously described were spread on nutrient agar plates and incubated for two hours to allow for phenomic lag (4,5). At the end of this period, all plates were overlayed with 0.7% agar containing 0.5 mg of streptomycin per ml and incubated for two days. Results of a typical experiment are shown in Table I. The appearance of control and experimental plates at the completion of the experiment is illustrated in Fig. 2. All streptomycin resistant clones were streaked on EMB xylose plates and were found to remain xylose negative. Phage lysates prepared from streptomycin sensitive as well as xylose negative strains were used as controls in earlier experiments and served to indicate that only factors present in the genotype of the donor strain could be transferred.

Using standard cell suspensions, an attempt was made to relate the number of cells transduced for the xylose marker to the number of phage particles used in treatment. The semilogarithmic graph (Fig. 3) demonstrates a straight line relationship between numbers of transduced positives and numbers of phage particles. The possible presence of a transforming factor liberated by the action of the phage and comparable to the genetically active desoxyribonucleic acid preparations reported by Hotchkiss(6) and many other investigators, was determined by means of a desoxyribonuclease preparation (25 mg/ml).* Results obtained after treatment of the phage lysates with this enzyme indicated no effect on the transducing ability of the lysates.

The experiments set forth here have been repeated with another of the Vi phages (Phage K). Other Vi phages in the series as well as other genetic factor transductions are now being examined.

Discussion. One might interpret the remarkable findings of Zinder and Lederberg (1) as a form of transformation using bacteriophages, symbiotic in the donor cells, which after appropriate treatment become part of the genetic constitution of the recipient cells. Whether the phage is the intrinsic transferring agent or merely serves as the carrier of the genetically active material has not as yet been clearly established.

The experiments reported here indicate that phage lysates of one typhoid strain can have a genetic effect on other strains in the series. It is pertinent at this point, to consider the origin of these Vi typing phages and their

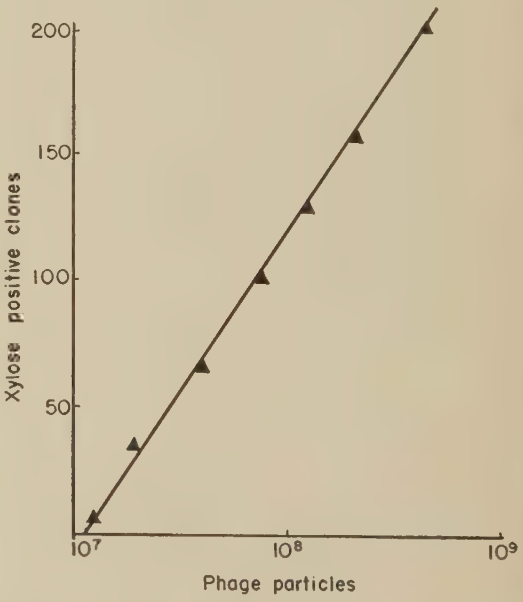


FIG. 3. *Effect of varying number of phage particles.* 10^8 cells of *S. typhosa* strain 643 treated with varying amounts of Vi phage E_1 and plated on EMB xylose agar. Xylose positive clones were counted after 48 hr.

---

* Desoxyribonuclease preparations were tested for activity on purified thymus nucleic acid (kindly supplied by Dr. A. Saz) prior to use in experimental procedures.

practical applications. Craigie and Yen(7) developed the Vi bacteriophage method of typing strains of *S. typhosa* by starting with one phage and through a mechanism of 'phage adaptation' produced a series of specific Vi phages. These phages are now used in the routine typing of typhoid strains according to a revised scheme and standardized technic suggested by Craigie and Felix(8). Felix and Anderson(9) refer to a classification of strains of typhoid on the basis of differential fermentation of l-arabinose and xylose and state that a xylose negative strain retained this biochemical characteristic during more than eight years maintenance in the laboratory. While the obvious genetic significance of transduction by phage is of paramount importance, since the situation presents many problems with far-reaching implications, it is nevertheless worthwhile to be aware of the practical problems likely to be encountered (see Felix and Anderson(9)). It is possible to conceive of symbiotic relationships in the Vi phage series wherein a number of phages may be carried by typhoid strains, for Rountree(10) has shown that a single bacterial strain can carry as many as 5 different latent phages. As far as the geneticist is concerned, the attempts at 'phage adaptation' in the production of the Vi phage typing series by Craigie and Yen(7) may afford an unparalleled tool for the study of symbiotic and lysogenic relationships between bacteria and phage. The presence of the Vi antigen in strains of non-typhoid organisms such as *Escherichia coli, S. ballerup* and *S. paratyphi C* offers the opportunity for possible inter-strain transductions and perhaps, ultimately, an explanation for the presence of this antigen in these strains. Further studies with these objectives in mind are now being conducted in this laboratory, along with attempts at the elucidation of the underlying mechanisms involved.

Summary. Lysates prepared by the action of specific Vi phage E_1 on *S. typhosa* strain Ty2 possess the ability to transfer (transduce) single hereditary traits to other Vi strains of *S. typhosa*. Fermentative ability and drug resistance have been transferred to strains lacking these factors. These experiments have been repeated using Vi phage type K with the same results. The data obtained showed considerable similarity to the findings of Zinder and Lederberg with the advantage that induction of lysogenicity is not a necessary factor in the initial preparation of active filtrates. The practical and genetic implications of this system and further avenues of analysis have been discussed.

---

1. Zinder, N. D., and Lederberg, J., *J. Bact.*, 1952, v64, 679.

2. Bertani, G., and Weigle, J. J., *J. Bact.*, 1953, v65, 113.

3. Adams, M., *Methods in Medical Research*, 1950, v2, 1.

4. Davis, B. D., *Experientia*, 1950, v6, 41.

5. Demerec, M., and Cahn, E., *J. Bact.*, 1953, v65, 27.

6. Hotchkiss, R. D., *Cold Spring Harbor Symposia Quant. Biol.*, 1951, v16, 457.

7. Craigie, J., and Yen, C. H., *Can. Pub. Hlth. J.*, 1938, v29, 448, 484.

8. Craigie, J., and Felix, A., *Lancet*, 1947, v1, 823.

9. Felix, A., and Anderson, E. J., *J. Hyg.*, 1951, v49, 349.

10. Rountree, P. M., *J. Gen. Microbiol.*, 1949, v3, 153.

Received May 18, 1953. P.S.E.B.M., 1953, v83.

Laboratory Transmission of Fibromas (Shope) in Cottontail Rabbits by Means of Fleas and Mosquitoes. (20339)

LAWRENCE KILHAM* AND PAUL A. WOKE.†

From the Department of Health, Education, and Welfare, Public Health Service, National Microbiological Institute, National Institutes of Health, Bethesda, Md.

Transmission of myxoma virus by mosquitoes, demonstrated by Aragao(1) in Brazil and recently in further detail by Fenner, Day and Woodroofe(2) in Australia, suggests that fibroma virus(3), a related agent, might be similarly transmitted. As will be shown *Aedes aegypti* proves to be a good vector of fibroma virus from cottontail to cottontail (*Sylvilagus sp.*). Fleas collected from wild cottontails also transmitted virus, although results were less striking. These findings indicate the probable importance of biting insects as vectors of this disease, the natural transmission of which is imperfectly understood.

Materials and methods. Virus: Fibroma virus employed for insect transmission was originally recovered from a wild cottontail captured at the Patuxent Research Refuge, Laurel, Md., and kindly given to us by Dr. Carlton M. Herman. Passages were made by intracutaneous inoculation of cottontail rabbits. Fibromas used for insect transmission were from 2 cottontails inoculated 48 to 63 days previously. The OA strain of Shope fibroma virus was used in serum neutralization tests. *Rabbits*: Cottontail rabbits were obtained from dealers in Kansas and Arkansas. Serum neutralization tests were performed in white domestic rabbits. Rabbits of both types were prepared for experimentation by removal of hair from the sides with clippers. *Neutralization Tests*: Sera from cottontails used in transmission experiments were tested for specific neutralizing antibodies after inactivation by heating for 30 minutes at 56°C. Undiluted serum was mixed with equal volumes of OA fibroma virus, prepared so as to give final dilutions of 10^{-3} and 10^{-4} which represented 100 and 10 infecting doses in these tests. Serum-virus-mixtures were inoculated intracutaneously into white rabbits in amounts of 0.5 ml. Results were read in 5 days by noting presence or absence of fibromas. *Fleas*: Fleas were obtained from wild cottontails caught in box traps in the vicinity of Rockville, Md.,‡ and transported in muslin bags. No attempt was made to identify fleas during experimentation, but as many as possible were recovered for identification.§ Three species were involved: *Cediopsylla simplex* (Baker); *Odontopsyllus multispinosus* (Baker); and *Hoplopsyllus affinis* (Baker); the first 2 species predominated. *Mosquitoes*: The mosquitoes, *Aedes aegypti* (Linnaeus), were of an old established laboratory strain. The adults were fed on raisins and through cloth on 5% sucrose solution, and were maintained at approximately 28°C and 70% relative humidity. Unless otherwise stated, the mosquitoes were fed on hosts individually. Each mosquito was confined to a small space near the open end of a 20 mm diameter culture tube, and fed through gauze covering. Those allowed to take full blood meals were confined in like manner to 45 mm diameter tubes, in groups of 5.

Results. Transmission by pins. Fibromas were induced in 3 cottontail rabbits by sticking pins to a depth of a few mm into a fibroma on an infected cottontail and then pricking the skin of a normal animal in similar fashion. After this procedure there was conspicuous oozing of blood from the fibromas but not from normal skin. A large triangular pin led to tumors 1 to 2 cm in diameter which had a gross and microscopic appearance characteristic of Shope fibroma, although they developed a crust and were regressing within 30 days. Finer pins of stainless steel induced smaller tumors on 2 additional rabbits. One cottontail died accidentally, but both of the others developed specific neutralizing antibodies along with growth of the fibromas. These preliminary experiments gave encouragement that insect transmission might succeed.

---

* Laboratory of Infectious Diseases.

† Laboratory of Tropical Diseases.

‡ The animals were loaned to us by the Maryland Inland Fish and Game Commission.

§ Fleas were identified by C. F. W. Muesebeck, Bureau of Entomology and Plant Quarantine, U. S. Department of Agriculture.

Transmission by fleas: Four cottontails were used in experiments in which flea transmission was attempted by 2 different methods. In initial experiments about 8 fleas were placed in gauze covered glass tubes (4 cm in diameter) which were then inverted over a fibroma allowing the fleas to feed through the open end. As gauze seemed to interfere with feeding it was later dispensed with and fleas were recovered with a suction tube to prevent escape at the termination of feeding. After a delay of 3 or 4 hours, fleas previously fed on an infected rabbit were then held over the skin of a normal cottontail. Each feeding episode lasted about 30 minutes, during which time about half of the fleas appeared to feed. None of these experiments in which the fleas were confined to tubes during both feedings was successful. Recipient cottontails developed neither lesions nor immunity. Successful results were obtained when a fourth cottontail was placed in a flea-proof cage together with fleas which had previously fed on a fibroma. A total of 25 fleas was used on successive days and some were recovered, refed on fibromas, and replaced in the cage. Two and a half weeks later numerous scabby thickenings appeared on the skin of the cottontail, especially at bases of both ears, on one shoulder, and on both outer thighs. These lesions regressed in several weeks at which time serum from this animal, which had previously been negative, neutralized over 100 infective doses of OA fibroma virus. Subsequent to bleeding, the rabbit was challenged with virulent fibroma virus and found to be immune. Although biopsies were not obtained, we have not encountered such lesions on rabbits infested with fleas. As discussed below, it seems possible that flea bites induce a shallower type of lesion than the more penetrating bites of mosquitoes.

Transmission by mosquitoes. In a preliminary experiment, 6 tubes with 10 mosquitoes each were held for a partial feeding on a fibroma (cottontail No. 343) and then immediately afterwards on the skin of a normal cottontail for completion of the feeding. Four other tubes of partially fed mosquitoes were kept 24 hours before being held on the same rabbit. Six fibromas, the first appearing in 8 days, resulted from the immediate transfer and 2 from the 24-hour delayed transfer, after interrupted feedings on the fibroma. These tumors enlarged to a maximum of 2 cm in diameter, remaining undiminished in size after 80 days. Specific neutralizing antibodies against the OA strain of Shope fibroma virus appeared in this rabbit within 4 weeks. Mutual interference in feeding resulted when many mosquitoes were held in a single tube. In later experiments mosquitoes were kept in individual tubes, which facilitated observations on the number of mosquitoes actually feeding on both fibroma and recipient cottontails.

The efficiency of *Aedes aegypti* as a vector of fibroma virus was demonstrated over various time intervals in a second set of controlled feeding experiments (Table I). Each day's experiment was performed on a fresh cottontail and was limited to mosquitoes which had had only one previous blood meal on a fibroma. This initial feeding was interrupted in the case of mosquitoes refed immediately,

TABLE I. Results of Single Feedings of Mosquitoes *(Aedes aegypti)** on Recipient Cottontails after Initial Feeding on Cottontail Fibroma.

Initial blood meal	Interval before feeding on recipient	No. mosquitoes feeding on recipient	No. fibromas transmitted	Ratio of No. fibromas transmitted to No. mosquitoes feeding
Interrupted	Immediate	15	18	1.20
"	24 hr	17	15	.88
"	4 days	32	18	.56
Full	1 wk	24	16	.67
"	2 "	16	13	.81

* Mosquitoes 5 days of age at time of initial feeding.

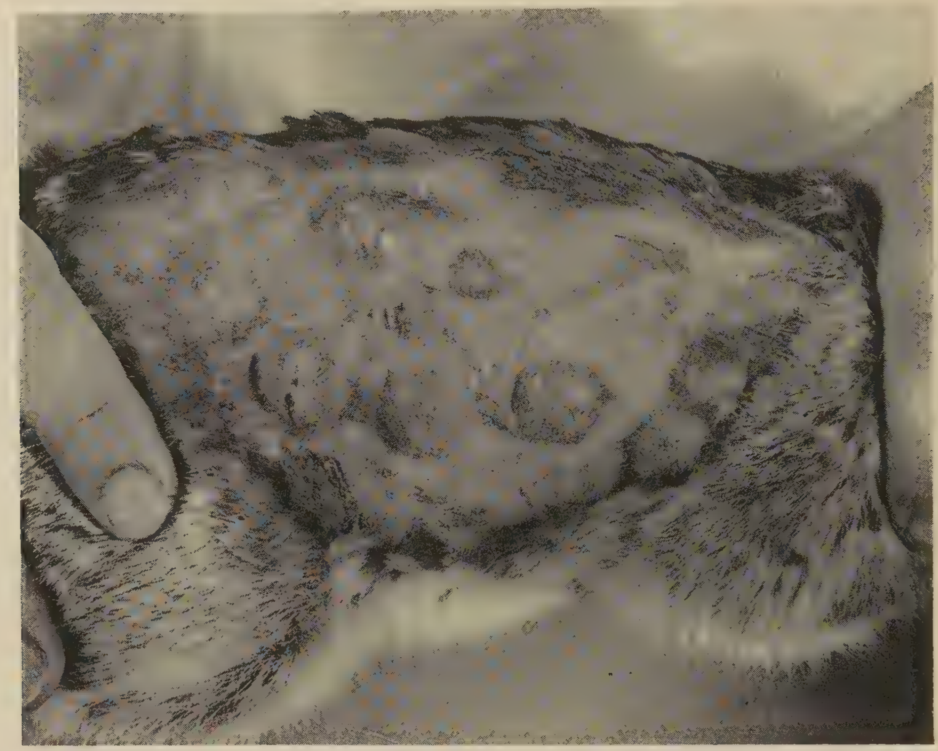


FIG. 1. Fibromas transmitted to cottontails by mosquitoes 14 days after full blood meal on a fibroma. Photo taken 35 days after mosquitoes had bitten.

in 24 hours, and in 4 days. Mosquitoes refed at 7 and 14 days had had a full bloodmeal on fibroma. As shown in Table I best results were obtained when 18 fibromas resulted from immediate interrupted feeding of 15 mosquitoes. As some of these vectors probably probed several times in the course of a single feeding, they may have inoculated fibroma virus in more than one spot. A finding of interest was that mosquitoes transmitted virus approximately as well in 2 weeks (13 fibromas resulted from exposure to 16 mosquitoes) as they did in 24 hours (15 positive out of 17 bites). Transmission of fibromas was somewhat less efficient in intervening periods, with only 18 of 32 bites being positive at 4 days and 16 of 24 being positive at 7 days after the initial feeding. In a control experiment, 5 mosquitoes fed on areas of unaffected skin 3 inches away from fibromas of an afflicted cottontail. No fibromas resulted when these mosquitoes were interrupted in their feeding and refed immediately on a normal cottontail. It should be pointed out that mosquitoes fed through cloth and on cotton soaked in sugar solution as well as on raisins during intervals between rabbit blood-meals. Gross appearances of fibromas 40 days after mosquito inoculation were not uniform for different time intervals. Those resulting from immediate interrupted feeding remained full and round with minimal scab formation. On the other hand, fibromas developing after the 24 hour, the 4 and 7 day intervals, became flat in 4 to 5 weeks due to formation of a thick dark scab, which had fallen off by 40 days leaving a small scar. Fibromas resulting from mosquitoes partially fed 2 weeks previously were in marked contrast to the preceding, remaining full and round, without scabbing. Many of them were 2 cm in diameter and bore a close resemblance to naturally occurring fibromas.

In a further experiment a normal cottontail with no fur removed was placed in a mosquito-proofed culture chamber. Twenty-four 19-day old mosquitoes were released into this chamber immediately following a partial

TABLE II. Persistence of Capacity to Transmit Fibroma Virus Shown by *Aedes aegypti** in the Course of Repeated Interrupted Feeding.

Intervals between initial feeding and successive bites on recipients		No. mosquitoes biting recipient	No. fibromas transmitted	Ratio of No. of fibromas transmitted to No. of mosquitoes biting
Days				
1	A.M.	13	6	.46
	P.M.	13	6	.46
2		8	2	.25
3	A.M.	8	1	.13
	P.M.	8	2	.25
7		5	None	0

* Mosquitoes 20 days old at time of initial feeding.

blood-meal on a fibroma. All mosquitoes had opportunities to feed on the enclosed rabbit overnight and 10 of them to feed over the following day and night. Two weeks later 2 fibromas had developed, one on the upper eyelid and a second one at the inner canthus of the same eye. No tumors were located elsewhere on the body of this animal. Specific neutralizing antibodies developed in the serum during the disease.

A final experiment was performed to determine how long individual mosquitoes might continue to transmit fibromas in the course of successive interrupted feedings. As shown in Table II, 12 fibromas were induced on a fresh cottontail by 2 separate feedings of 13 mosquitoes within 24 hours of feeding on a fibroma. After 4 previous bites, 8 mosquitoes induced 2 fibromas on the afternoon of the third day, but 5 of these 8, biting a fresh cottontail on the 7th day, failed to transmit fibroma virus. In gross appearance tumors induced in this experiment underwent scab formation and regression within 40 days, being comparable to tumors transmitted in 24 hours, 4 and 7 days in the experiment described above (Table I). The same fibroma-bearing cottontail (No. 337) was used for initial feeding in all experiments indicated in Tables I, II, which were carried on more or less concomitantly.

The lower percentage of transmission attained in the final experiment may be accounted for in part by the fact that the mosquitoes were allowed only to pierce rather than to feed for an extended time. An unusually high mortality in this group of mosquitoes, however, complicates interpretation of results obtained. Deaths of the last 5 mosquitoes terminated this experiment.

Discussion. Success of other investigators (1,2) in transmitting myxomatosis by means of mosquitoes suggested that similar results might be obtained with Shope fibroma. There is evidence indicating that myxoma and fibroma are closely related if not variants of the same virus. Thus, one may be transformed into the other(4), there is a high degree of reciprocal cross immunity(5), both are chronic benign diseases of cottontail rabbits (Sylvilagus), one in South America (1) and one in North America(3), and finally both can be transmitted by similar vectors. Fenner has recently reviewed classification of these 2 agents(6). As fibroma virus is transmissible by pin pricks, it would seem that mechanical transfer might be possible for a variety of biting insects. Philip(7) found that triatomid bugs would transmit fibroma among domestic rabbits. Domestic rabbits however are not a natural host and react differently to fibroma virus than do cottontails. We have therefore confined experiments to the natural host (Sylvilagus), hoping that results might give some clue to modes of transmission operative in nature. Fleas, an abundant parasite on most wild cottontails, were used in initial transmission experiments. Positive bites were obtained only when fleas, after feeding on a fibroma, were released on an enclosed cottontail. Resulting lesions were crusted and pox-like, leading to as high a level of circulating antibodies in the course of infection as did typical fibromas induced by mosquitoes in other cottontails. It is conceivable that flea bites are less penetrating than those of mosquitoes and thus result in a more superficial type of lesion. Similar lesions have been found in wild grey squirrels (*Sciurus carolinensis*) in association with specific neutralizing antibodies and flea infestation(8). These animals may also have typical fibromas(9).

Aedes aegypti is an efficient vector of fibroma, transmitting virus immediately and up to 2 weeks in a high proportion of bites. Transmission is presumably mechanical for the first few days. It is difficult, however, to understand how virus can persist on the proboscis of a mosquito for 2 weeks, during which time the insect has been feeding on raisins or through cloth on sugar water. That virus may get rubbed off is suggested by the final experiment. Here, although numbers were small, 5 successive partial feedings in 3 days led to a reduction in numbers of tumors induced. In general, results obtained in transmitting fibroma parallel those obtained by other investigators in mosquito transmission of myxoma(1,2) and of fowl pox(10-12). This similarity of behavior gives support to the hypothesis suggested by Fenner(6) that these disease agents are related. It would seem to us that further experimentation should be carried out to prove or to disprove whether these viruses proliferate in the transmitting mosquitoes. Fenner and Day(11), however, believe that myxoma and pox viruses are spread by mechanical means, the mosquito serving figuratively as "flying pins".

After mosquitoes carrying fibroma virus were released to feed at will on a recipient cottontail, the animal subsequently developed fibromas close to one eye. Aragao(1), mentions that the only myxoma tumors found on a wild Brazilian cottontail were in this location. Natural fibromas are usually found on the feet and legs of wild cottontails, the reason for which is unknown. One may speculate that either some native mosquitoes are attracted to the shorter fur of these areas or that some different vector is involved, as Shope(13) has suggested. There would seem to be several factors which would favor transmission of fibromas in nature. One factor is the long persistence of virus in fibromas(8). A second factor of possible importance is that these highly vascular, superficial tumors are readily fed upon by at least 2 blood sucking insects. Thirdly, as we have shown, at least one type of vector can transmit the virus for a considerable period of time after a single feeding. To fully investigate natural modes of transmission one would need to find an area in which cottontail fibromas are epizootic; a situation which, in our experience, has been difficult to locate.

In summarizing present experiments, it would appear that mosquitoes may function as more than "flying pins", especially if one considers the variation of appearance in fibromas 5 to 6 weeks after their inception. Those transmitted immediately and in 2 weeks were larger and persisted with little change after 40 days while those from intervening periods became scabby and regressed early, so that only scars remained after 5 and a half weeks. The latter tumors were comparable to those transmitted by pins whereas the former, especially those transmitted after 2 weeks, bore a close resemblance to naturally occurring fibromas. Possibly virus transmitted in intermediate intervals was less in amount or attenuated by exposure on the mosquito proboscis. A possible explanation of the larger, more characteristic appearance of the other tumors might be that virus was fresh and fully virulent on immediate transmission. If virus growth took place in the mosquito after more than a week, virus transmitted in 2 weeks might again be fully virulent. All mosquitoes used in these particular experiments were of the same lot and had their initial feeding on the same fibroma-bearing cottontail. Although full interpretation of phenomena encountered on a small number of cottontails is difficult, it is not unreasonable to suppose that *Aedes aegypti* may serve initially as a mechanical and subsequently as a biological vector of fibroma virus. Continued experiments are being carried out in an effort to further test this hypothesis.

Summary. 1. Fibroma virus may be transmitted from cottontail to cottontail by means of pin pricks, fleas found on cottontails, and by the mosquito *Aedes aegypti* (Linnaeus). 2. *Aedes aegypti* transmits fibroma virus under favorable circumstances, in as high a propor-

tion of bites at 2 weeks following initial term feeding as at 24 hours. 3. Virus carrying mosquitoes induced fibromas on the eyelid and close to the eye of one cottontail when given the opportunity to feed at will. 4. Possible mechanisms involved in mosquito transmission are discussed.

---

1. Aragao, H. B., *Mem. Inst. Oswaldo Crus,* 1943, v38, 93.

2. Fenner, F., Day, M. F., and Woodroofe, G. M., *Aust. J. Exp. Biol.,* 1952, v30, 139.

3. Shope, R. E., *J. Exp. Med.,* 1932, v56, 793.

4. Berry, G. F., and Dedrick, H. M., *J. Bact.,* 1936, v31, 50.

5. Shope, R. E., *J. Exp. Med.,* 1936, v63, 33.

6. Fenner, F., *Nature,* 1953, v171, 562.

7. Philip, C. B., *J. Parasit.,* 1942, v28, 395.

8. Kilham, L., in preparation.

9. Kilham, L., Herman, C. M., and Fisher, E. R., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 298.

10. Kligler, I. J., Muckenfuss, R. S., and Rivers, T. M., *J. Exp. Med.,* 1929, v49, 649.

11. Kligler, I. J., and Ashner, M., *Brit. J. Exp. Path.,* 1929, v10, 347.

12. Fenner, F., and Day, M. F., *Nature,* 1952, v170, 204.

13. Shope, R. E., *Cornell Vet.,* 1951, v41, 181.

Received May 22, 1953. P.S.E.B.M., 1953, v83.

Effect of Calcium Salt of Versene upon Metabolism of Plutonium in the Rat.* (20340)

JOSEPH G. HAMILTON AND KENNETH G. SCOTT.

From the Divisions of Medical Physics, Experimental Medicine, Radiology, Crocker Laboratory, University of California, Berkeley and San Francisco.

Because of the potential hazard of the fission products and fissionable materials to individuals who may have been exposed to them, considerable interest has been devoted to the pharmacology of these radioactive substances as well as the development of methods for removing them from the body. Any procedure which results in a greater than normal elimination of these materials from the body has value from two points of view, first it can reduce the ultimate hazard to the contaminated individual, and second it allows a more accurate estimation of the degree of contamination which may be suspected in personnel exposed to such hazards.

One of the compounds which has received attention as a decontaminating agent has been the sodium and calcium salts of ethylene diamine tetra-acetic acid, later referred to as Versene. The pharmacology of this organic compound has been reviewed in detail by the Bersworth Chemical Co.(1). Popovici *et al.* (2) investigated the control of serum calcium levels using the sodium and calcium salts of Versene. They demonstrated that the sodium salt was many times more toxic than the calcium salt due to the fact that the former compound combined with the serum calcium and produced death by hypocalcemia. Cohn *et al.* (3) has recently investigated the effects of the calcium salt upon the deposition and retention of radio-yttrium using rats as the experimental animals. In their studies the Versene salt and radio-yttrium were administered intraperitoneally, either shortly before or after radio-yttrium was given. The animals were sacrificed at intervals up to 5 days and a very marked diminution of skeletal uptake was observed in the treated animals. The greatest effect was noted in those animals which had been pretreated and the skeletal uptake was approximately 1/10 that of the control groups and the content in the liver was decreased by a factor of approximately 20. Earlier studies at this laboratory have demonstrated that oral administration of the calcium salt of Versene increased the urinary excretion of plutonium(4).

Methods. The dosage of Versene and mode of administration is summarized in each table. Wherever administered, Versene was given as the calcium salt at a pH of 7. Female rats weighing from 200 to 310 g were used. The treated animals and controls received Pu^{238}. The dosage varied in the several experiments from 0.18 to 0.09 μc. The plutonium was administered at plutonyl nitrate in the presence of 0.01 M bromate which kept it in $+6$ valance state and 0.1 M citrate which forms a complex with plutonium thereby preventing the formation of a plutonium colloid. The 92-year Pu^{238} was used rather than the longer lived Pu^{239} in order to secure a higher degree of specific activity and thus approach a plutonium concentration as to the number of atoms that might be present in human beings who may have been exposed to this radioelement and who might have of the order of 1 μg of the longer lived isotope Pu^{239} stored in their body. In all 5 experiments the treated and control animals were maintained in metabolism cages, each cage containing from 3 to 5 rats. The urine and feces were collected separately. The organs and tissues removed at the time of sacrifice were assayed for plutonium content and these included: spleen, blood, kidney, gastrointestinal tract, muscle, skin, injected leg of the fourth group as well as the "balance" of the rat. The balance represented the ashed remainder of the rat after it had been skinned, the skeleton removed from the ash and the calculated amount of plutonium in blood, muscle and skeleton subtracted from the total plutonium content of the balance. In the case of the animals which received plutonium by intramuscular injection an additional correction was made since the injected leg was removed at the proximal end of the femur. Thus the balance may be presumed to represent structures such as glandular, lymphoid, connective tissue and cartilage. Most of the tissues were assayed for their plutonium content by the technic which has been described in detail earlier by Scott *et al.*(5). In other cases specimens containing relatively large amounts of calcium such as the skeleton, balance, liver, skin, urine and feces necessitated the use of a second assay technic in which the chelating agent Thienyl Trifloroacetone was used to separate the plutonium from the tissue ash. This chemical, commonly called TTA, will effect a quantitative removal of plutonium from animal residues when the following method is employed. *TTA method for separation of plutonium from tissue ash.* 1. Add 50 cc of 2 *N* HNO_3 plus 0.1 M hydroxylamine hydrochloride solution to ashed sample and stand for at least 2 hours. 2. Put 10 cc sample in centrifuge cone. 3. Add 3 cc of $La(NO_3)_3$ without stirring (20 mg $La(NO_3)_3$ per cc). 4. Add 10 cc of 12 M HF and stir. 5. Centrifuge for 5 minutes at 2000 rpm. 6. Pour off supernatant when precipitate is well packed down. 7. Add 10 cc of 1.5 M HF to cone and stir precipitate. 8. Centrifuge again 5 minutes at 2000 rpm and pour off supernatant when precipitate is well packed down. 9. Stir precipitate to break it up. Add 25 cc of 3.5 M $Al(NO_3)_3$ and stir until precipitate dissolves. 10. Add .25 cc of 0.1 M $NaNO_2$. 11. Stir and let stand at least 15 minutes before pouring into a separatory funnel. 12. Add 10 cc of TTA solution containing 25 g of TTA in 500 cc of benzene and shake 10 minutes. 13. Let stand one hour and drain off bottom layer of liquid. 14. Wash twice with distilled water. 15. Add 10 cc of 8 M HNO_3 and shake 15 minutes. 16. Let stand for 5 minutes. 17. Drain product and 8 M HNO_3 rinse from separatory funnel into porcelain, gold at platinum dish. 18. Evaporate to dryness and count.

---

* This document is based on work performed under the auspices of the Atomic Energy Commission.

TABLE I. Distribution of 0.18 μc of Pu^{238} at 1 and 15 Days after I.V. Inj. to Control and Versene Treated Rats. The former given 2 mg Versene I.V. at time of inj. plus 3.5% in diet 24 hr prior to Pu^{238} inj. 3 animals in each group. Values expressed are % of the dose per g wet wt and % excreted.

	1 day		15 days	
	Control	Versene	Control	Versene
Muscle	.06	.05	.01	.01
Skeleton	2.33	2.38	2.98	3.07
Urine	.89	3.37	1.9	10.5
Feces	2.23	2.36	16.0	17.6

Results. The results obtained from the first 2 experiments are summarized in Table I, and show that the oral administration of Versene is not an effective way of removing plutonium from the rat. The simultaneous injection of Versene intravenously at a dosage of 8 mg per kilo of body weight had little effect on altering the eventual distribution of plutonium in the

TABLE II. Distribution of 0.09 μc of Pu^{238}, 15 Days after I.V. Inj. in Tissues of Control and Versene Treated Rats. Values expressed as % of dose/organ and g wet wt. Corrected to 100% recovery. Average recoveries were 88.7% for the 10 animals in control group and 89.7% for the 9 animals in Versene group. 186 mg of Versene given I.P. at time of inj.; 3.5% in diet starting 24 hr prior to Pu^{238} inj.

	Control				Versene			
	% per organ	Mean* stand. error	% per g	Mean* stand. error	% per organ	Mean* stand. error	% per g	Mean* stand. error
Spleen	.60 ±	.06	.67 ±	.08	.17 ±	.05	.20 ±	.09
Blood	.40	.04	.02	<.01	.28	.05	.02	<.01
Liver	8.77	.52	.89	.04	3.59	.38	.39	.04
Kidney	1.37	.13	.76	.07	.50	.08	.26	.05
G.I. tract	.88	.07	—	—	.32	.04	—	—
Skeleton	55.4	.92	2.62	.12	30.6	.20	1.35	.09
Muscle	1.39	.13	.01	<.01	.71	.10	<.01	—
Balance	2.70	.15	—	—	.90	.10	—	—
Skin	1.48	.12	.03	<.01	.51	.06	.01	<.01
Urine	6.16	.19	—	—	50.6	1.28	—	—
Feces	20.9	.77	—	—	11.7	.29	—	—

* Mean stand. error $\pm = \sqrt{\frac{\Sigma \text{dev}^2}{n(n-1)}}$, for Tables II, III and IV.

TABLE III. Distribution of 0.11 μc of Pu^{238}, 15 Days after I.M. Inj. in Tissues of Control and Versene Treated Rats. Values expressed as % of dose/organ and g wet wt, corrected to 100% recovery and representing only plutonium absorbed from inj. site. Average recoveries were 90.9% for the 9 animals in control group and 86.7% for the 10 rats in treated group. 140 mg Versene given I.P. for first 5 days, none for following 3 days, 155 mg daily for next 7 days.

	Control				Versene			
	% per organ	Mean* stand. error	% per g	Mean* stand. error	% per organ	Mean* stand. error	% per g	Mean* stand. error
Spleen	.44 ±	.05	.54 ±	.03	.20 ±	.05	.28 ±	.08
Blood	.47	.04	<.04	—	.16	.02	.01	<.01
Liver	6.50	.52	.97	.09	2.12	.16	.27	.01
Kidney	.94	.06	.61	.05	.38	.03	.25	<.01
G.I. tract	.80	.12	—	—	.31	.03	—	—
Skeleton	63.6	.70	4.39	.17	34.4	1.77	2.04	.12
Muscle	1.21	.13	.01	<.01	.66	.06	<.01	—
Balance	4.29	.57	—	—	1.08	.19	—	—
Skin	1.03	.10	.04	<.01	.36	.03	.01	<.01
Urine	4.10	.15	—	—	47.1	2.18	—	—
Feces	16.6	.71	—	—	13.2	1.03	—	—
Left leg	24.4	—	—	—	3.79	—	—	—

* See footnote, Table II.

tissues in animals which were also being fed 3.5% Versene. Although the group summarized in Table I had an increased urinary plutonium excretion no significant reduction was observed in the amount of plutonium found in the skeleton, and muscle.

The parenteral administration of large amounts of Versene to rats following a dose of plutonium is capable of reducing the plutonium stored in most tissues of the body. When plutonium was administered intravenously and Versene given parenterally, a significant decrease of plutonium in the tissues of rats sacrificed 15 days following plutonium administration was observed. These data are summarized in Table II. There was nearly an 8-fold increase of urinary excretion with the administration of Versene. The amount of plutonium found in liver, muscle, skin and balance of these treated animals was significantly less than that in the control. The amount of plutonium retained by the skeleton was nearly one-half that of the untreated control animals. A comparison of these data with

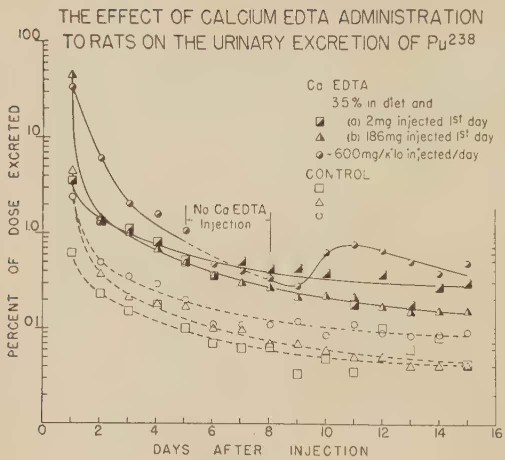


FIG. 1.

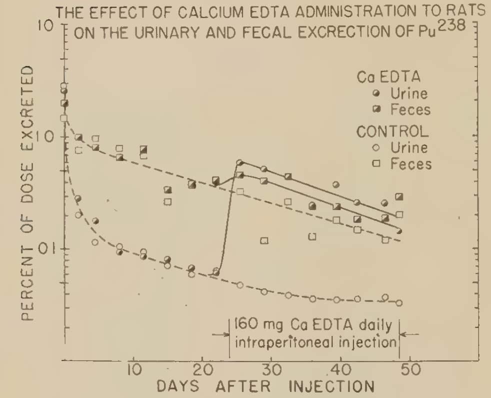


FIG. 2.

those obtained in the first 2 experiments indicates that the greater effect was apparently due to the administration of large amounts of Versene parenterally as compared to the action of this compound when present in the diet, since the treated animals in Groups 2 and 3 had the same diet.

The results obtained by the multiple injections of large amounts of Versene in animals on a normal diet are summarized in Table III. In general the results are very similar to those noted in the third experiment. An interesting observation in the fourth experiment was the effect of Versene upon the increased absorption of plutonium from the site of the injection, which was by the intramuscular route. Fig. 1 presents graphically the effect of Versene upon the urinary excretion of plutonium for the second, third, and fourth experiments. There was apparently some increase of urinary excretion in all 3 groups and it seemed greatest in the fourth group.

Fig. 2 indicates that the parenteral administration of Versene nearly a month after plutonium was given produced quite a prompt and definite increase of its excretion. The complete distribution of plutonium in the tissues in the fifth experiment is shown in Table IV. The fecal excretion of the Versene-treated and control animals was nearly the same. The depletion of plutonium in the tissues and organs was minimal. The greatest effects were seen with spleen, skin and balance. Here the plutonium content was reduced by nearly a factor of two. The skeletal content of plutonium was significantly lower in the Versene-treated animals but the reduction was not as great as that observed in the soft tissue.

Discussion. These studies indicate that prolonged oral administrations of Versene at levels that approached a significant degree of toxicity did not produce a pronounced depletion of plutonium in the soft tissues and had little or no effect upon the skeletal content of this element. The parenteral administration of small amounts of Versene likewise appears to be productive of little effect. The parenteral administration of large amounts of Versene at the same time plutonium was given appeared to decrease the skeletal content to a significant degree and also increase the absorption from the intramuscular site of injection. The latter observation is of interest since a very probable route of exposure of an individual to plutonium might be by subcutaneous or intramuscular introduction into the body. It would be of interest to determine if the parenteral administration of Versene is capable of reducing the plutonium content in the lungs following its inhalation as an aerosol and more particularly in a chemical state which can be absorbed and enter the blood stream, Scott *et al.*(5).

A comparison of these studies with those of Cohn *et al.*(3) are of interest in that the effect they observed was much greater than that noted in the experiments described in this paper. However, it should be kept in mind that in the studies by Cohn *et al.*(3) the

TABLE IV. Distribution of 0.12 μc of Pu^{238} in Tissues of the Rat 50 Days after I.V. Inj. Values expressed as % of dose/organ and g wet wt and corrected to 100% recovery. Average recovery of the 10 control animals was 82.3% and 90.0% for Versene treated animals. 162 mg Versene given daily by I.P. from 25th to 50th day following Pu inj.

	Control				Versene			
	% per organ	Mean* stand. error	% per g	Mean* stand. error	% per organ	Mean* stand. error	% per g	Mean* stand. error
Spleen	.91	± .05	1.60	± .17	.45	± .07	.67	± .10
Blood	.15	<.01	.01	<.01	.14	<.01	.01	<.01
Liver	5.25	.44	.63	.07	4.90	.67	.47	.06
Kidney	.68	.07	.37	.05	.65	.02	.32	.01
G.I. tract	.29	.04	—	—	.16	.02	—	—
Skeleton	61.2	1.13	3.29	.16	52.0	1.08	2.74	.09
Muscle	1.06	.18	.01	<.01	.80	.09	<.01	—
Balance	3.68	.15	—	—	1.51	.18	—	—
Skin	.98	.04	.03	<.01	.54	.11	.02	<.01
Urine	6.33	.40	—	—	15.2	.40	—	—
Feces	19.5	1.11	—	—	24.1	.68	—	—

* See footnote, Table II.

Versene compounds as well as the radio-yttrium were given by intraperitoneal injection. It is entirely possible that in their studies a large fraction of the radio-yttrium was chelated before it was absorbed. Thus their experiments and those described here are not entirely comparable.

With current information available it would appear that Versene is of very limited practical values for the treatment of chronic plutonium poisoning. The relatively small increase of urinary excretion after this radio-element has been deposited in the skeleton together with the large amount of Versene which appears necessary would seem valid reasons for not giving this substance as a practical therapeutic agent. Moreover, in the rat, the administration of Versene necessary to secure a significant amount of urinary excretion is in the toxic range. The immediate parenteral use of large amounts of Versene following the accidental entry of plutonium into the body through cuts or abrasions would appear to offer some promise. Before such attempts might be made, careful consideration should be given to the problems of acute and chronic toxicity of Versene compounds in man.

Conclusion. 1. Oral administration of Versene in the rat at a toxic range did not produce a marked depletion of plutonium in soft tissues and had but little effect upon skeletal content. 2. When given parenterally large amounts of Versene depleted the skeletal content to a significant degree. 3. Under these conditions the absorption of plutonium from the intramuscular site of injection was increased. 4. These experiments indicate that it would appear that the use of Versene in chronic human plutonium poisoning offers little promise. 5. The prompt administration of Versene in large amounts might conceivably be of benefit when plutonium has entered the body by cuts or abrasions.

The authors wish to express their appreciation for the technical assistance of Miss Margaret Gee, Miss Marilyn Hemenway and Miss Helen Johnson.

---

1. Bersworth Chemical Co., Framingham, Mass., Technical Bulletin, *Pharmacology, Physiology, Biochemistry and Toxicity of Versenes.*

2. Popovici, Antoinette, Geschickter, Charles, Reinovsky, Albert and Rubin, Martin, PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 415.

3. Cohn, S. H., Gong, J. K., and Fishler, M. C. *Studies on the Treatment of Internal Radioactive Contamination. II. Prevention of Deposition of a Fission Product by Salts of Ethylene Diamine Tetra-Acetic Acid.* USNRDL-358.

4. Foreman, Harry and Hamilton, Joseph G. *The Use of Chelating Agents for Accelerating Excretion of Radioelements.* UCRL-1351.

5. Scott, Kenneth G., Axelrod, Dorothy, Crowley, Josephine and Hamilton, Joseph G., *Arch. Path.*, 1949, v48, 31.

Received March 24, 1953. P.S.E.B.M., 1953, v83.

Local Effects of Epinephrine on the Cerebral Cortex. (20341)

BRUNO MINZ,* ANTOINE G. REMOND,† AND W. S. MCCULLOCH.
From the Department of Psychiatry, University of Illinois College of Medicine, Chicago, Ill.

Recent work has shown(1) that small doses of epinephrine, injected intravenously or intra-arterially into the cat, prolong the duration of electrically induced seizures and shorten the period of extinction after electrical stimulation. It seemed of interest to consider the effects of locally applied epinephrine on the excitability of the cerebral cortex.

These experiments were carried out on the adult monkey (*Macacus rhesus*) under moderate Dial anesthesia (40 mg/kg). The anterior part of the hemispheres was exposed; the dura was opened and the motor arm areas located on both sides. A bipolar electrode was placed on each hemisphere with the negative pole in the arm area just anterior to the central sulcus, the positive pole, posterior. The selected areas were stimulated with a thyratron stimulator, producing condenser discharges of 1 msec. time constant at 50 impulses per second for 2 seconds. Stimulation of both areas set up equal movements in the corresponding hands. The voltage employed (around 6 volts) was just sufficient to elicit the chosen movement when there were no after-effects from previous stimulation. Cortical excitability was evaluated by recording cortical and muscular responses to a pair of stimuli, the second separated from the first by increasing intervals of time. The pairs of stimuli were applied to the hemispheres alternately 3 minutes apart. In order to discover whether changes in the cortical responses of these stimuli after local application of epinephrine accounted for the augmentation of motor responses, we recorded: 1) the electrocorticogram from the stimulating electrodes, 2) the electromyogram from the groups of muscles involved, 3) the movement.

Results. A) *Control curves.* The first stimulus evoked a movement in the opposite hand; the second stimulus, initially given after a very short time interval, regularly elicited cortical and muscular after-discharges. The interval between the stimuli was then progressively increased until the second stimulus failed to produce any after-effects. This interval was generally between 4 and 8 seconds. Different intervals were found for electrocorticographic, myographic and mechanical measurements of after-discharges. The first can be recorded in the absence of the others. With a slightly higher voltage, cortical after-discharges may be accompanied by a small myographic pattern characterized by rhythmic discharges of motor units. With still higher voltages, the myographic record becomes complex and movement always occurs.

B) *The effect of epinephrine.* After recording control curves of all responses and their facilitation for the series of intervals, a small piece of filter-paper soaked in epinephrine solution (1/1000) was placed on one cortical arm area. The electrodes were replaced, the positive, posterior as before, the negative, anterior, on the filter-paper. Cortical activity was then immediately recorded and the excitability tested as before by determining the responses to pairs of stimuli, alternating between treated and untreated hemispheres, at progressively increasing intervals (Fig. 1).

The epinephrine employed in 3 experiments was a product going under the trade name of "Suprarenin-Bitartrate"—Winthrop; it contained synthetic 1-epinephrine bitartrate in isotonic solution, 0.5% chloretone and 0.2% sodium bisulfite. The immediate effect of this solution applied on the cortex was a considerable decrease in amplitude with disappearance of high frequencies of the corticogram from the treated area (Fig. 2). In spite of this apparent depression, stimuli applied to the treated area produced after-discharges at noticeably longer intervals than on the untreated side (Fig. 3). Finally, the first stimulus, which originally caused only the selected muscular movement, was late in the experi-

---

* Visiting Research Professor of Pharmacology, Univ. of Illinois.

† Research Associate, Univ. of Illinois, with a grant of The Cerebral Palsy Foundation.

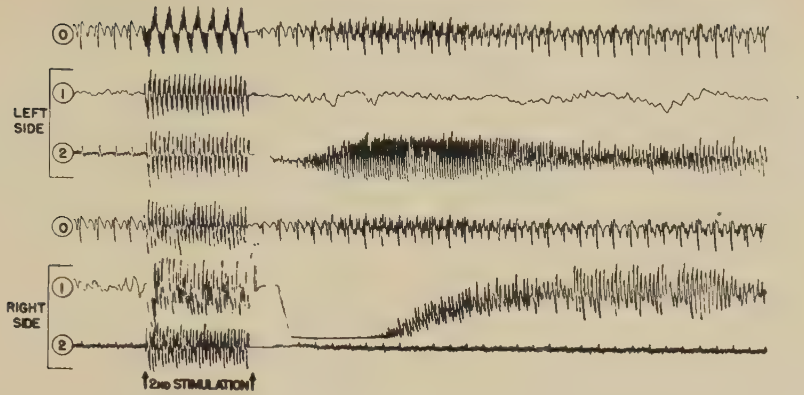
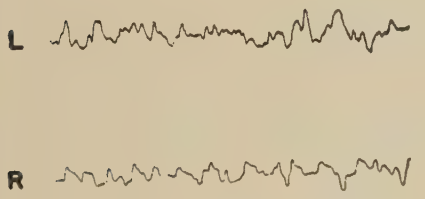
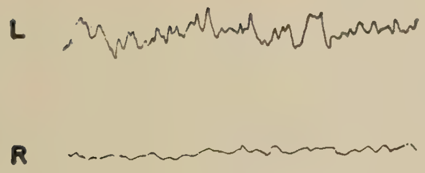


FIG. 1. Effect of a second cortical stimulation on the right side. (1 msec. 50 impulses per sec., 6 volts during 2 sec.) First stimulation, 4 sec. before, is not shown. 0—electrocardiogram; 1—corticogram of the hand motor cortex; 2—myogram of ipsilateral hand muscles. After stimulation, there follows a transitory alternation of record (1) on the right side (cortical after-discharge) and of record (2) on the left side (myographic after-discharge).



CONTROL



AFTER EPINEPHRINE

FIG. 2. Corticograms of right and left cerebral cortex before and immediately after local application of epinephrine to the right side.

ment infrequently followed by after-discharge. Occasionally, this last effect was not limited to the treated side but appeared, after a considerable delay, on the control side, in which case there was always a parallel modification of the electrocardiogram, with bradycardia and arrhythmia, suggesting passage of epinephrine into the circulation. Three other experiments performed with a freshly prepared solution of epinephrine with 1/1000 barbiturate and three experiments with a similar epinephrine solution with 1/1000 chlorhydrate instead of barbiturate failed to show the cortical depression observed in our first experiments.‡ It is thus possible that that phenomenon may be due to chloretone, though similar effects have been obtained with eserine and acetylcholine on the cat's cortex by Miller, Stavraky and Wooton(2). Local application of these epinephrine solutions elicited, however, in all our experiments the

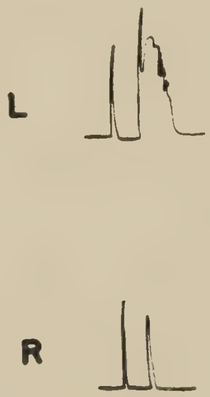


FIG. 3. Kymographic record of the same movement on both sides. On the left after application of epinephrine the movement following a right cortical stimulation is complicated by an after-discharge after the second stimulation.

---

‡ From previous work in this laboratory it is known that bisulfite in the concentrations employed here does not depress cortical activity.

appearance of muscular after-discharges for longer intervals than on the untreated side; we further found an increase in duration of cortical after-discharges and a decrease of about 30 percent of the voltage necessary to produce movement. This observation may be of interest in connection with McCulloch's recent findings(3) that eserine and atropine which generally sensitize and inhibit central acetylcholine effects and which have been found to prolong and to shorten respectively the period of cortical facilitation, do not produce any change in the cortical threshold to electrical stimulation.

Local application of an epinephrine solution 1/10,000 as well as that of isotonic saline, was ineffective in our control experiments.

Conclusions. 1. On the basis of these results, it may be concluded that epinephrine stimulates neuronal aggregates in the cerebral cortex. 2. The local effect is marked by an increase in duration and intensity of the period of over-excitation subsequent to a stimulus eliciting movement, and it is characterized by the appearance of after-discharges of cortex and muscle after stimuli just sufficient to produce movement.

1. Minz, B., and Domino, E., *Fed. Proc.*, 1952, v11, 376.
2. Miller, F. R., Stavraky, G. W., and Wooton, G. A., *J. Neurophysiol.*, 1940, v3, 131.
3. McCulloch, W. S., *Biology of Mental Health and Disease*, Milbank Memorial Fund Symposium, Paul B. Hoeber Inc., 1952.

Received March 31, 1953. P.S.E.B.M., 1953, v83.

Experimental Poliomyelitis. Studies in Mice with Human Gamma Globulin. (20342)

SIDNEY J. KLEIN AND JOHN M. ADAMS.
(With the technical assistance of Barbara J. Spargo.)

From the Departments of Pathology and Investigative Medicine, Veterans Administration Hospital, Long Beach, Calif., and the Departments of Pediatrics and Infectious Diseases, School of Medicine, University of California, Los Angeles.

Recent preliminary evaluation of gamma globulin as a prophylactic agent for poliomyelitis in children by Hammon, Coriell, Wehrle, Klimt, and Stokes(1), demonstrated significant temporary protection. Experimental study of gamma globulin to determine dosage, time of administration, and duration of passive protection, becomes a matter of considerable importance.

The data in the present report deal exclusively with studies in mice which offer a convenient though limited tool for investigating certain quantitative aspects of the problem. Bodian(2) protected cynomolgus monkeys with doses of gamma globulin as small as 0.1 ml/kg of body weight when the poliomyelitis virus was fed immediately after the administration of gamma globulin. Adams and his co-workers(3,4) found that when cynomolgus monkeys were tonsillectomized, much larger doses were required for protection. A dose of 2.0 ml/lb inoculated 24 hours prior to the feeding of virus gave complete protection. Rhodes and Clark(5) reported an *in vitro* neutralization titer of approximately $10^{-3.5}$ for gamma globulin tested against Lansing virus in mice. Hammon, Cheever, and Sather(6) obtained some protection against poliomyelitis in suckling mice with gamma globulin in 0.05 ml/lb dose.

Methods. A rodent-adapted Y-SK strain* of poliomyelitis virus was employed. After 4 intracerebral passages through mice, virus-infected brains and spinal cords of 7 mice were harvested aseptically and emulsified in distilled water to a 10% suspension. This suspension was titrated by intracerebral inoculation of white mice. The LD_{50} dose calculated at 21 days according to the Reed-Muench(7) formula, was $10^{-3.35}$. The 10% suspension was stored in a deep freeze with dry ice and was used as source of virus for all experiments in this report. Samples were thawed as needed and appropriately diluted in sterile distilled water to contain 50 LD_{50} doses of virus in a 0.03 ml intracerebral inoculum, as estimated by titration at the start of this series of experiments. Each experiment was controlled for virus potency by a group of mice inoculated with virus alone. Mice were examined for paralysis or death daily for a period of at least 5 weeks. The gamma globulin employed was a concentrate of pooled human plasma gamma globulin provided by the American National Red Cross (Immune Serum Globulin Human 160 mg of gamma globulin per cc Squibb). *In vitro* neutralization tests were performed in the usual way(8). Constant amounts of virus were mixed with an equal volume of dilutions of gamma globulin in sterile saline. The mixtures were kept at 37°C for 1 hour and in the refrigerator overnight, then inoculated intracerebrally into groups of 8 white mice.

---

* Kindly provided by J. R. Paul.

Results. In vitro neutralization. The concentration of gamma globulin required for 50% survival at 30 days, calculated according to the Reed-Muench method of accumulation totals, was $10^{-3.05}$. Since the mice in this experiment averaged 22.5 g weight, 0.015 ml of this concentration of gamma globulin (1/1130) corresponded to a dosage of 0.0006 ml/kg body weight.

In vivo neutralization. In this experiment, gamma globulin in varying amounts was inoculated intraperitoneally into mice 1 day prior to the intracerebral test dose of virus. A divided dose was used only in the group receiving the largest amount of gamma globulin, where the second injection was given 48 hours later. The 30-day results are shown in Table I. The gamma globulin dosage was computed on the basis of average mouse weight of 22.5 g in this series. The 50% neutralization dose of gamma globulin, when given 1 day before virus inoculation, was calculated as 8.5 ml/kg. This was more than 14,000 times the 0.0006 ml/kg dose which sufficed for 50% neutralization of the same virus dose *in vitro*.

TABLE I. Protective Effect of Gamma Globulin *In vivo* when Given One Day before Poliomyelitis Virus.

	Gamma globulin (ml/kg)						
	50	20	10	5	.5	.05	0
Mice dead or paralyzed at 30 days	1/7	4/8	6/8	4/8	7/8	8/8	6/8
Cumulative mortality index*	.047	.243	.461	.633	.930	1.000	

* Inclusion of 2 additional doses of gamma globulin between the critical values of 5 ml/kg and 50 ml/kg introduced the factor of unequal log-dose intervals. Irwin's generalization of the Reed-Muench method was therefore used to calculate the 50% neutralization dose of gamma globulin(9).
50% neutralization dose = 8.5 ml/kg.

Duration of protection with gamma globulin. The purpose of this experiment was to determine the optimal interval for administration of gamma globulin prior to injection of virus. Use of an excessively high dose of gamma globulin was to be avoided therefore. Three-week-old mice were divided into 11 groups. All groups except the control received 0.09 ml of gamma globulin intraperitoneally but on a staggered schedule. When all mice were injected simultaneously with the constant challenge dose of virus 2 months later, the interval between the prophylactic and infective inoculations varied from 2 months to one-half hour in the different groups as indicated in Table II. When the amount of gamma globulin was referred to average body weight of 22 g on the day of infection, the dosage was calculated as 4.1 ml/kg. Table II shows the results at different preinfection intervals. Since there was no single correct date for terminating the experiment, Table II records the results obtained at 21, 30, and 38 days after infection. After 38 days, new cases among the mice had virtually ceased. At 21 days after infection, the groups which had received gamma globulin up to 4 days prior to infection, all showed survival of one-half or more mice. None of the other groups showed significantly greater survival than did the untreated controls (17%). At 38 days, only the 4-day pretreated group continued to show any large number of survivors (5 of 8). The chi-square test for significance of the difference between the 4-day group and control group, gave a

TABLE II. Protective Effect of Gamma Globulin* *In vivo* when Given at Varying Intervals before Poliomyelitis Virus.

Days after infection	Mice dead or paralyzed	Time gamma globulin preceded virus										Control: no gamma globulin
		Days										
		60	46	32	14	7	4	3	2	1	½ hr	
21	No.	4/4	4/4	6/9	8/9	9/11	1/8	2/9	5/10	4/10	5/12	10/12
	%	100	100	67	89	82	13	22	50	40	42	83
30	No.			9/9	8/9	10/11	3/8	5/9	7/10	5/10	9/12	11/12
	%			100	89	91	38	56	70	50	75	92
38	No.				8/9	10/11	3/8	8/9	7/10	6/10	10/12	11/12
	%				89	91	38	89	70	60	83	92
		Repeat experiment										
21	No.			9/15†	8/13	5/10	2/10	4/10	3/10	4/10	9/11	8/13
	%			60	62	50	20	40	30	40	82	62
30	No.			13/15	10/13	5/10	4/10	5/10	3/10	5/10	10/11	10/13
	%			87	77	50	40	50	30	50	91	77

* 0.09 ml intraper.

† This group received gamma globulin 28 days before virus.

"P" value of 0.02-0.05 (Yates' correction for small numbers). The significance of this result is increased by the observation that none of 15 control groups which had received the same virus suspension and dose in the course of these experiments, showed more than 25% survival at 30 days. In the total of 129 control mice, mortality at 30 days was 91%. Especially noteworthy was the complete absence of protection in the 7-day pretreated group. Three additional groups of 12 mice received the same dose of gamma globulin 1, 4, or 7 days after virus inoculation. The first two groups showed no protection; the last showed only equivocal protection, and are not included in the table.

Repetition of previous experiment. To test the validity of the observations of the last experiment, a similar protocol was carried out. In this experiment, average mouse weight on day of infection was 32.2 g. The same gamma globulin dose of 0.09 ml intraperitoneally was, therefore, equivalent to 2.8 ml/kg. The virus suspension was inoculated in 1/45 dilution as before. The results are also recorded in Table II. At 21 days after infection, the groups which had received gamma globulin, 4, 3, 2, or 1 days before infection, all showed lower mortality rates than did the control group. Amongst these, the 4-day group again appeared to have an advantage.

Plotting the curves of mortality rate against time for each group, did not reveal more than a transitory advantage of questionable significance for the 4-day group as against the 3, 2, 1-day groups. The 7-day pretreated group showed mortality rate curve in close parallel to the control group for the first 20 days. The results then diverged, indicating some possible protection in this group. The 14-day, 28-day, and ½-hour pretreated groups showed mortality rates equal to or greater than the control group throughout the period of observation.

Application of the chi-square test to the 30-day readings in Table II, indicated in both experiments a probability of 0.01-0.02 that the observed variations in mortality might be due to chance alone. These data also showed a statistically significant reduction in mortality for the combined 1, 2, 3 and 4-day groups, contrasted with all other groups. Combined mortalities were 54% vs 90%, and 42.5% vs 77.4% ($P < 0.001$ in both series).

Discussion. The findings of several investigators have suggested that passive immunity to poliomyelitis obtained with practicable doses of gamma globulin was only of few weeks' duration(1,10-13). In our experiments on duration of passive protection, all groups were infected simultaneously with the identical virus suspension, and special effort was made to determine the endpoint of the protection period. The 0.09 ml dose of gamma globulin was equivalent to about 3-4 ml/kg body weight, yet the mice showed complete loss of protection against intracerebral challenge within the brief space of 7 days in one experiment, and within 2 weeks in the second experiment.

It is a matter of some interest that gamma globulin given intraperitoneally in mice may require as much as four days to achieve maximal protection. The explanation for the lag-period is not entirely clear. Kramer(11) found that when convalescent serum was inoculated into mice intraperitoneally, the level of poliomyelitis neutralizing antibody in the circulation was high the next day and fell sharply within the following 3 days. However, Morgan, Schlesinger, and Olitsky(14) and Kramer(11) presented evidence that the concentration of neutralizing antibody in the central nervous system—the *locus minoris resistentiae*—was a more important criterion of immunity to intracerebral inoculation of virus than was circulating antibody. Appearance of antibody in the brain and spinal fluid lagged behind antibody in the blood(14).

It is important then to consider that experiments on passive protection with gamma globulin or immune serum administered a customary 24 hours before intracerebral virus challenge, may not constitute a test under conditions of maximal prophylactic effect.

Summary. 1. The dosage of gamma globulin required to protect mice against poliomyelitis virus intracerebrally was several thousand times the amount which was effective for *in vitro* neutralization. 2. The prophylactic effect of gamma globulin was maximal with a dose of 4 ml/kg intraperitoneally when given 4 days before inoculation of virus intracerebrally; at 7 to 14 days protection had apparently disappeared.

1. Hammon, W. M., Coriell, L. L., Wehrle, P. F., Klimt, C. R., and Stokes, J., Jr., *J. Am. Med. Assn.*, 1952, v150, 757.

2. Bodian, D., *Am. J. Hyg.*, 1952, v56, 78.

3. Adams, J. M., Carpenter, C. M., French, J. D., Pressman, J. J., Smith, J. L., and Klein, S. J., *Laryngoscope*, 1951, v61, 1010.

4. Adams, J. M., Boak, R. A., Carpenter, C. M., French, J. D., Klein, S. J., Pressman, J. J., and Smith, J. L., *J. Lab. Clin. Med.*, 1953, v41, 142.

5. Rhodes, A. J., and Clark, E. M., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 379.

6. Hammon, W. M., Cheever, F. S., and Sather, G. E., PROC. SOC. EXP. BIOL. AND MED., 1952, v80, 150.

7. Reed, L. J., and Muench, M., *Am. J. Hyg.*, 1938, v27, 493.

8. Paul, J. R., and Riordan, J. R., *Am. J. Hyg.*, 1950, v52, 202.

9. Armitage, P., and Allen, J., *J. Hyg.*, 1950, v48, 298.

10. Bodian, D., *Am. J. Hyg.*, 1951, v54, 132.

11. Kramer, S. D., *J. Immunol.*, 1943, v47, 277.

12. Wood, W., Clark, E. M., McKendrey, J. B. J., and Rhodes, A. J., PROC. SOC. EXP. BIOL. AND MED., 1952, v80, 522.

13. Dixon, F. J., Talmadge, D., and Maurer, P. H., *Fed. Proc.*, 1952, v11, 466.

14. Morgan, I. M., Schlesinger, R. W., and Olitsky, P. K., *J. Exp. Med.*, 1942, v76, 357.

Received April 13, 1953. P.S.E.B.M., 1953, v83.

Electrophoretic Plasma Protein Patterns in Families with Hemophilia.*

(20343)

PETER BERNFELD, MARIO STEFANINI, RICHARD D. BERKOWITZ, AND FRANCIS B. HENNESSEY.

From the Cancer Research and Cancer Control Unit, Departments of Surgery, of Biochemistry, and of Medicine, Tufts College Medical School, Boston, Mass.

The appearance of an electrophoretic anomaly in a group of hemorrhagic diseases has been described in an earlier paper(1). This anomaly, which was called α_x-globulin, represents one of the few instances in which the electrophoretic patterns are typical for a given disease or for a certain group of diseases. The detection of this anomaly was made possible by the thorough morphologic study of the electrophoretic patterns(2) rather than by the customary determination of the relative concentrations of the individual protein components. The anomaly is more pronounced in the rising boundaries than in the descending boundaries. It possesses all of the following characteristics: 1. The appearance of a sharply pointed peak (α_x) which migrates somewhat faster than the usual α_2-globulin peak; the mobility of this α_x-globulin in the rising boundaries ranges between 4.3 and 4.6 x 10^{-5} cm^2 volt^{-1} sec^{-1}, in veronal citrate buffer of pH 8.6 and ionic strength $\mu = 0.1$. 2. The disappearance of the usual α_2- and/or α_3-globulin peaks, *i.e.*, there is no peak perceptible between the α_x- and the β-globulin. 3. The pronounced asymmetric shape of the α_x-peak with a very steep slope towards the albumin, but with only a gradual decline towards the β-globulin. Although it cannot be ascertained whether the α_x-globulin peak actually represents a particular type of protein absent in normal and in most pathological plasma, or merely an electrophoretic artifact, it is possible to calculate from this peak a relative concentration of protein. Accordingly, the α_x-globulin, whenever it appears, represents between 6 and 15% of the total plasma protein. To date, in a population in which blood diseases are represented in an unusually high proportion, the α_x-anomaly has been found in 102 cases out of a total of 440 individuals studied by electrophoresis. With very few exceptions, this electrophoretic characteristic occurs only in patients with certain hemorrhagic and certain allergic diseases. This anomaly is found with great constancy in hemophilia, in idiopathic thrombocytopenia of the acute variety(3), and in Henoch-Schönlein purpura which is probably on an allergic basis.

---

* This study has been supported by an Institutional Grant of the American Cancer Society, New York City.

In the present paper the α_x-anomaly is shown to occur also in the plasma of members of hemophilic families although they may not be bleeders themselves. A relationship between the presence of an α_x-peak and the prothrombin utilization in the plasma of these individuals is described. The prothrombin utilization which is known to be high in normal blood but low in hemophilic blood, is usually high in mixtures of known hemophilic with normal blood. When the blood of a known hemophiliac is mixed with the blood of a member of a hemophilic family having an α_x-peak, however, a low prothrombin utilization may be observed.

Methods. Electrophoretic experiments were carried out as described in a companion paper(2). *Test for the detection of deficiency of anti-hemophilic protein* (*prothrombin utilization*): The plasma of a known hemophiliac is first obtained. Blood is added to 1/10 volume of 0.2 M sodium citrate and centrifuged at 2000 r.p.m. for 10 minutes. Plasma from the individual who is being investigated is collected similarly. Equal volumes of known hemophilic plasma and plasma under investi-

TABLE I. Prothrombin Utilization in Members of Hemophilic Families.

Name	Sex	Age	Family relationship	Bleeder	Prothrombin time (sec.) of clotted mixture of plasma under investigation and known hemophilic plasma	α_x-globulin
			Family M.			
Mary M.	♀	56	Mother	—	59	—
Sally M.	♀	20	Children of Mrs. Mary M.	—	55	—
Thomas M.	♂	18		—	13	±*
Martin M.	♂	15		+	9	+
Jack M.	♂	11		—	12	+
Nancy M.	♀	10		—	14	+
Patricia M.	♀	9		—	15	±*
			Family S.			
Mary S.	♀	42	Mother	—	69	—
William S.	♂	12	Children of Mrs. Mary S.	+	15	+
Joan S.	♀	10		—	22	+
Gerald S.	♂	7		+	11	+

* ± designates a doubtful α_x-globulin.

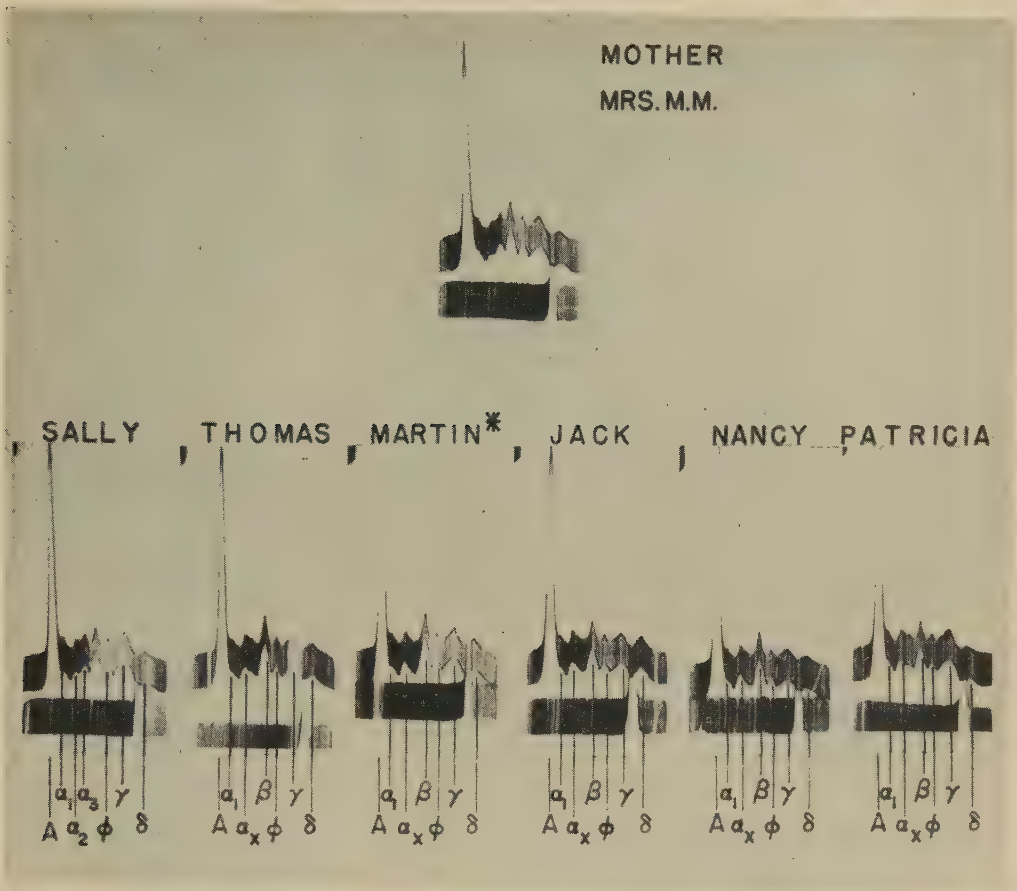


FIG. 1. Electrophoretic plasma protein patterns of Family M. * designates a bleeder. A = albumin; α_1, α_2, α_3, β, and γ = the corresponding globulins; $\alpha_x = \alpha_x$-anomaly; ϕ = fibrinogen; and δ = remainder of δ-salt anomaly; the lower lines represent the beginning gradients at time $t = 0$.

gation are mixed. The mixture is then recalcified by the addition of 1/10 volume of 0.2 M calcium chloride and, one hour after completion of the clotting, the serum is separated and its prothrombin activity determined according to the method described by Stefanini and Crosby(4).

Results. A total of 14 individuals with active hemophilia have been studied. An α_x-globulin anomaly was present in the plasma of all 14 individuals.

Furthermore, the plasma of the members of two hemophilic families which included three of the above mentioned "bleeders" has been studied. The electrophoretic patterns of the plasma of 11 members of these families are shown in Fig. 1 and 2.

The α_x-anomaly is present in the plasma of the 3 "bleeders", W.S., G.S., and M.M., but also in the plasma of 3 other family members, J.S., J.M., and N.M., who are not bleeders, including 2 females, J. S. and N.M. A somewhat doubtful α_x-peak is present in 2 other individuals, T.M., and P.M., in these 2 cases, the peak is believed to be a doubtful α_x-globulin because of the presence of another peak in these patterns with the mobility of the usual α_2-globulins.

The results of the prothrombin utilization (prothrombin time of the clotted mixture of plasma of each person and known hemophilic plasma) are given in Table I. A low prothrombin utilization is indicated by a low prothrombin time; a value below 25 seconds is considered to be typical for active hemophilia. It is obvious that the low prothrombin utiliza-

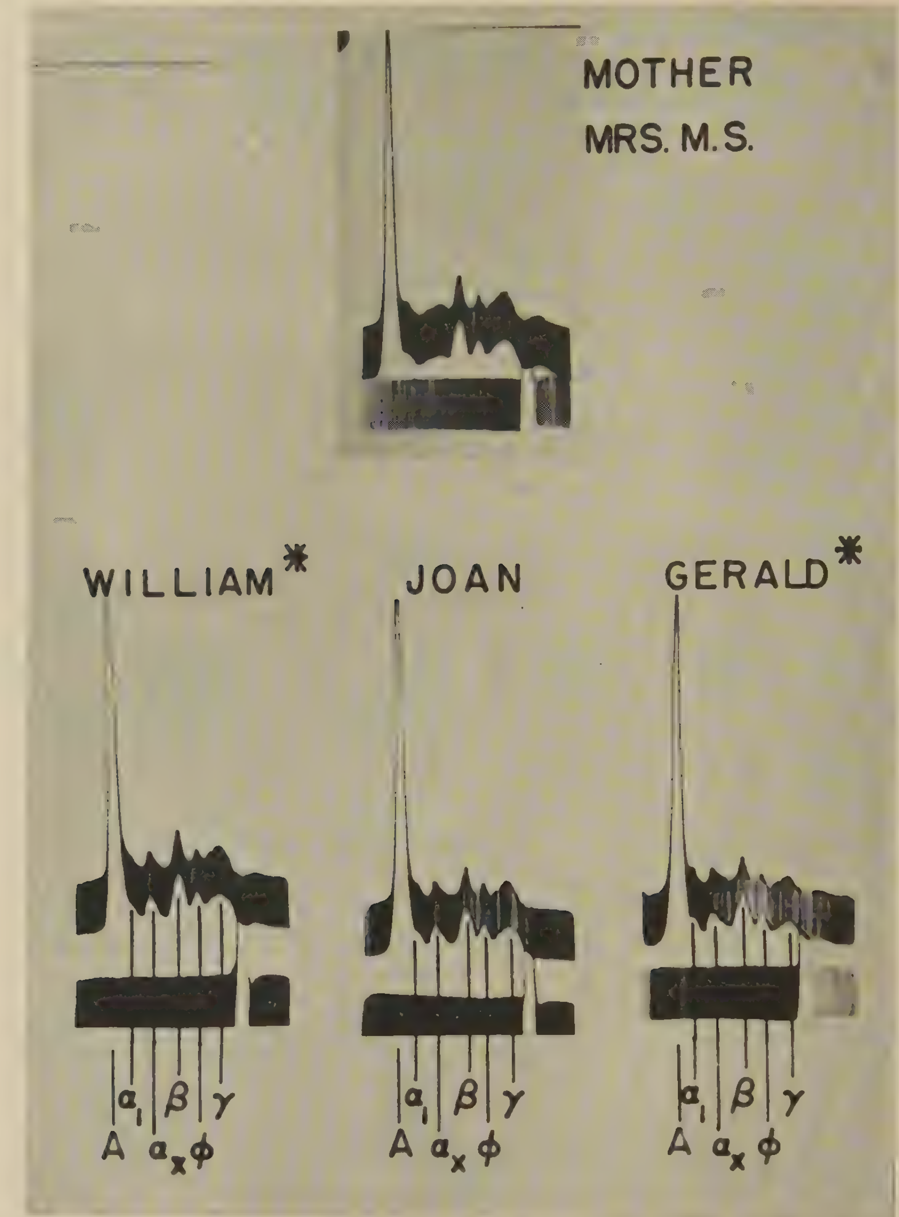


FIG. 2. Electrophoretic plasma protein patterns of Family S. * designates a bleeder. A = albumin; α_1, β and γ = the corresponding globulins; α_x = α_x-anomaly; ϕ = fibrinogen; the lower lines represent the beginning gradients at time t = 0.

tion not only appears in the plasma of the three bleeders, but also occurs in all those non-bleeding family members whose plasmas show an electrophoretic α_x-anomaly.

The appearance of an α_x-globulin has also been found in three nonbleeding members from two other hemophilic families.

Discussion. There is an obvious relationship between the appearance of an α_x-globulin in the electrophoretic patterns and a low prothrombin utilization in the plasma of members of hemophilic families. Both phenomena may be unrelated, however, to active bleeding itself.

From this study it is not possible to state the degree to which this electrophoretic anomaly is related to the heredity of hemophilia, nor is it possible to predict from the electrophoretic patterns whether a woman will be a carrier of hemophilia or not. From the 7 members of hemophilic families, who are not bleeders themselves but have the electrophoretic anomaly, 3 are females. It is possible that these women will be carriers of the disease. A woman may be a carrier of hemophilia, however, without showing the electrophoretic anomaly. This follows from the observation that the mothers of the two families presented in this paper do not show the α_x-anomaly, although both are believed to be the carrier of hemophilia, the disease being transmitted in both families to male descendants.

Summary. 1. An electrophoretic anomaly, called α_x-globulin, has been found in the plasma of 14 hemophiliacs, *i.e.*, in all of the active bleeders studied. 2. The same anomaly also has been observed in 8 members of hemophilic families who are not active bleeders (over 50% of this category), and who are not afflicted with any of the other diseases in which this anomaly has been found. 3. The electrophoretic anomaly is seen in female as well as in male subjects unlike the bleeding manifestation of hemophilia, found only in males. 4. There is a definite relationship between the prothrombin utilization and the appearance of the α_x-globulin anomaly in the plasma of members of hemophilic families.

1. Bernfeld, P., and Stefanini, M., PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 551.

2. Bernfeld, P., Donahue, V. M., and Homburger, F., PROC. SOC. EXP. BIOL. AND MED., in press.

3. Stefanini, M., Dameshek, W., and Bernfeld, P., *Clin. Research Proc.*, in press.

4. Stefanini, M., and Crosby, W. H., *Blood,* 1950, v5, 964.

Received April 27, 1953. P.S.E.B.M., 1953, v83.

Problems with Spontaneous Ectromelia (Mouse Pox) in a Virus Laboratory.* (20344)

JOSEPH L. MELNICK AND WILLIAM H. GAYLORD, JR.†

From the Section of Preventive Medicine, Yale University School of Medicine.

Since its discovery by Marchal(1), ectromelia virus, the causative agent of mouse-pox, has been recovered from several stocks of laboratory mice in Europe(2). Although mice have been reported in this country with a disease having similar signs, the virus has not been recovered from stock mice in the United States until recently when Trentin described an epizootic in the mouse colony of the Department of Anatomy at Yale(3). The conditions under which he isolated ectromelia virus are outlined in his paper. It was believed that the virus had been eliminated from the colony. However, we have recently observed a severe epizootic among our stock mice and have identified ectromelia virus as the causative agent. This report serves to indicate that the infection as late as February, 1953, was present among laboratory mice in the New Haven area.

The Outbreak. A number of non-specific deaths began to appear in the mouse colony of our laboratory in October and continued through the fall of 1952. During this period 200 pregnant mice arrived each week from 2 dealers. In addition, 200 to 300 were raised in New Haven each week for use when they reached the age of 4 weeks. For several months prior to the outbreak the newborn mice were being employed for isolating Coxsackie viruses from sewage and flies, and the adult mice chiefly for determining neutralizing antibodies against Lansing poliomyelitis virus. The total mouse population at any one time was a few thousand and rarely over 5000.

---

* Aided by a grant from the National Foundation for Infantile Paralysis.

† James Hudson Brown Memorial Fellow.

TABLE I. Cross Hemagglutination Inhibition of Ectromelia and Vaccinia Viruses.

Serum source	Serum titer against 5 units of: Ectromelia virus	Serum titer against 5 units of: Vaccinia virus
Mice convalescent from spontaneous ectromelia infection	80	40
" 10 days after inj. of ectromelia virus	40	<20
Rabbit 242, immunized against vaccinia	80	80
" 243, " " "	80	80
" (McCrae) immunized against vaccinia	160	160

The incidence of non-specific deaths became so high that it was not possible to carry out neutralization tests in mice which had been in the laboratory for a few weeks. Skin lesions were not noted during the early part of the epizootic. In November occasional mice were seen with swollen or necrotic legs. An attempt to pass an infectious agent from such legs as well as from the livers of these mice met with failure. (As the recipient mice had been in the stock colony for at least a month, they might have been immune.) In January, the epizootic increased in virulence; the number of deaths in the colony increased to well over 50% of uninoculated animals and the appearance of swollen legs, which often became necrotic, increased markedly. The hind limbs were involved with much greater frequency than the forelimbs. Lesions of the skin, generally of the legs but also of the hairless areas of the body proper, were noted.

Passage of an agent from sick mice in early January was successful, both in 4- and 12-week-old mice, newly arrived in the laboratory. Intraperitoneal inoculation of 10% suspension of either liver or leg suspensions resulted in death of recipient mice, after an incubation period of about 6 days. Moribund animals often contained increased peritoneal fluid, the livers were pale (fatty) and sometimes white necrotic foci could be found. Inoculation into the foot pad resulted in 4 to 5 days in a localized swelling which was followed by necrosis of the distal portion of the limb. Death usually followed in a few days, although some animals recovered. The virus grew readily in the chorioallantoic membrane of the chick embryo yielding pocks such as described for ectromelia(4). Suspensions of infected membranes readily agglutinated red blood cells selected from chickens whose cells had been found to agglutinate in the presence of vaccinia virus. In all respects observed the agent behaved like ectromelia virus(2). As no known ectromelia virus was in the laboratory, the virus was identified antigenically by its cross reactions with vaccinia. These consisted of cross neutralization tests in chick embryos and of cross hemagglutination inhibition tests(5). As the virus was identified as that of ectromelia, it will be henceforth referred to by name.

Cross hemagglutination inhibition tests.‡ *Viruses.* First passage ectromelia virus on the chorioallantois and a rabbit testicular passage line of the Levaditi vaccinia strain were used. Two-fold dilutions of virus were made in 5% rabbit serum in saline (10 x 75 mm test tubes) and to 0.2 ml of each dilution, there was added 0.2 ml of 0.8% suspension of chicken cells (chicken No. 683). Readings made at 60 minutes gave a titer of 1:320 units for ectromelia and 1:640 for vaccinia. In the test 5 units of each virus were employed, *i.e.*, for ectromelia a dilution of 1:64 and for vaccinia, 1:128.

Sera. Five sera were employed: 1) sera taken from mice convalescent from the natural disease (1-22-53); 2) sera taken from first passage mice 10 days after inoculation in the foot pad (1-29-53); 3) 4) and 5) sera from rabbits immunized against the Levaditi vaccinia virus, No. 242 (3-27-47), No. 243 (3-27-47), and McCrae (1-20-53), respectively. Serum was diluted in saline serially in twofold steps, 0.2 ml of each dilution distributed in 2 test tubes. Ectromelia virus was added to one set, and vaccinia virus to the other, in each case 5 hemagglutinating units being present in 0.2 ml. After mixing, 0.2 ml of 0.8% suspension of washed chicken cells were added to each tube and readings made 60 minutes later. The results are shown in Table I.

---

‡ Dr. J. F. McCrae kindly provided the chick cells, the vaccinia virus, and one of the vaccinia immune sera used in these tests.

The results show that the vaccinia sera inhibit hemagglutination by either virus to the same degree, the serum titers being 1:80 to 1:60. The ectromelia mouse sera yielded higher titers with the homologous virus. Thus one serum had a titer of 1:80 against ectromelia virus and only 1:40 against vaccinia. A less potent serum yielded a titer of 1:40 against ectromelia virus and failed to react with vaccinia virus at the lowest dilution tested, 1:20.

Cross neutralization tests in chick embryos. Chorioallantoic titrations were carried out in 12-day eggs using Burnet and Boake's modification of drawing the inoculum (0.1 ml) into the egg during the process of lowering the chorioallantoic membrane to form the base of the artificial air sac(5). Equal volumes of serial dilutions of virus and undiluted serum were mixed, held for one hour at room temperature, and 0.1 ml of each mixture inoculated into 4 eggs. Vaccinia-infected eggs were examined 48 hours later; ectromelia-infected eggs, 96 hours after inoculation.

The viruses in the test were first-passage foot pad suspension for ectromelia and the rabbit testicular suspension used above for vaccinia. Mice 10 days after inoculation of the foot pad suspension were the source of ectromelia serum, and a rabbit, No. 243 referred to above, immunized and bled in 1947 was the source of the vaccinia antiserum. The results are shown in Table II and indicate a cross relationship between the two viruses. The failure of the ectromelia serum to neutralize the homologous virus is probably an indication of the low antibody titer of this serum, for in the hemagglutination inhibition tests it had a titer of only 1:40 against the homologous virus and of less than 1:20 with vaccinia virus. The vaccinia antiserum neutralized both viruses.

TABLE II. Cross Neutralization Tests between Ectromelia and Vaccinia Viruses and Sera.

Serum	Virus titer	
	Ectromelia	Vaccinia
Ectromelia mice, 1-29-53	10^{-4}	10^{-6}
Vaccinia rabbit, No. 243, 3-27-47	10^{-2}	$<10^{-4}$
Saline control	10^{-4}	10^{-8}

Discussion. The virus isolated from the stock colony was identified as that of ectromelia by its biological properties and by its reciprocal relationship with vaccinia virus in serological reactions. Furthermore electron micrographs of thin sections of the chorioallantois of embryonated eggs infected with the local ectromelia strain have revealed virus particles of the pox group(8). The conditions under which the epizootic arose in our stock mice are unknown. Although the natural disease had appeared a year or so earlier in the stock mice of the Department of Anatomy, there is no known connection between mice or personnel handling mice in the two departments. However, as the virus is resistant to drying and subsequent storage at room temperature for 8 weeks(6), infected material may circulate through devious routes. One must also consider whether ectromelia virus was brought in from the outside. If this infection exists among wild mice(7), then it is conceivable that some specimens of sewage and flies which were inoculated into test mice may have contained the virus, although we believe that this possibility is remote. According to available information, the disease does not occur in the stock mice of either dealer from whom mice were obtained.

All mice in our laboratory were destroyed during the end of January and the beginning of February, 1953; all cages and bottles were sterilized; and the racks and room were washed down with 5% formalin. Virus harvests from mice infected with other agents during this period have also been destroyed, for ectromelia virus was undoubtedly present in some of the tissue suspensions stored in the frozen state. A new colony was brought in during February and to date the disease has not reappeared.

Summary. Trentin(3) has recently reported an outbreak of ectromelia (mouse pox) in his mouse colony at the Yale University School of Medicine. Another spontaneous epizootic in a different colony at the School is described in the present report. The virus was isolated and was shown to be related to vaccinia virus by cross hemagglutination tests and cross neutralization tests. The infected colony as well as harvests of other viral agents prepared from mice in the colony have been destroyed.

1. Marchal, J., *J. Path. Bact.*, 1930, v33, 713.
2. Literature reviewed by Fenner, F., *J. Immunol.*, 1949, v63, 341.
3. Trentin, J. J., *Science*, 1953, v117, 226; Trentin, J. J., and Briody, B. A., 1953, v117, 228.
4. Burnet, F. M., and Lush, D., *J. Path. Bact.*, 1936, v43, 105.
5. Burnet, F. M., and Boake, W. C., *J. Immunol.*, 1946, v53, 1.
6. Fenner, F., *Australian J. Exp. Biol. Med. Sci.*, 1947, v25, 327.
7. McGaughey, C. A., and Whitehead, R., *J. Path. Bact.*, 1933, v37, 253.
8. Gaylord, W. H., and Melnick, J. L., *J. Exp. Med.*, in press.

Received April 29, 1953. P.S.E.B.M., 1953, v83.

Intracellular Distribution of Digitoxin.* (20345)

SHIRLEY ST. GEORGE, MEYER FRIEDMAN, AND TADASHI ISHIDA.
From the Harold Brunn Institute, Mount Zion Hospital, San Francisco, Calif.

Recently we have demonstrated in various animals(1) that digitoxin readily leaves the blood stream to enter the various organs in quite significant amounts. However, the possible intracellular fate of digitoxin administered to the intact animal has never been determined because of the technical difficulties heretofore involved in such a study. Yet, in view of the uncertainty concerning both the site and mode of action of this glycoside, such information might be of considerable value. This is particularly so since the intracellular site of various enzymes now has been determined with a high degree of certainty.

On the introduction of the ultracentrifugal method for routinely isolating and obtaining intracellular components(2,3) and a microsensitive assay for the quantitative detection of digitoxin(4), it appeared possible that some information could be obtained concerning the intracellular fate of parenterally administered digitoxin. The results of a study using these technics are reported in this communication.

Methods. 1. *Separation and Collection of Intracellular Components.* The methods used, except for slight modifications, are those which have been described by Schneider and Hogeboom(3). The rats used were Long-Evans strain males, weighing 200 to 250 g. Rat liver was obtained five minutes after the intravenous injection of digitoxin (1 mcg. per gram of body weight) and rinsed in isotonic sucrose until the washings appeared free of gross blood. The liver was then forced through a tissue press into a beaker containing isotonic sucrose, the pulp weighed and sufficient sucrose solution added to prepare a 10% homogenate. Hearts were grossly dissected free of vessels and valves, weighed and minced with a scissor into the homogenizer containing sucrose. The homogenate was spun at 600 g for 10 minutes, the supernatant removed and the sediment washed, then respun again at the same speed and for the same period. The sediment obtained was identified by staining and microscopic study as the nuclear and debris fraction (Nw fraction)† The supernatant and washings were combined and spun at 5000 g for 10 minutes. The sediment was washed again and respun at 5000 g for 10 minutes. The sediment obtained was identified as the mitochondrial fraction (Mw2 fraction), the supernatant fluid identified as the fraction S_1 (no mitochondria were seen in this fluid) described by Schneider and Hogeboom(3). Similar fractions were obtained from the hearts of rats given the same quantity of digitoxin, except that an additional sedi-

---

* Aided by a grant from Eli Lilly and Co.

† The nomenclature used in labelling these fractions is that of Schneider and Hogeboom(3).

TABLE I. The Digitoxin Content of Liver and Heart Cell Fractions Immediately after Its Administration.

	Fraction Nw			Fraction Mw2			Fraction S_1		
		% of total in all fractions			% of total in all fractions			% of total in all fractions	
Total organ dig., µg/g	Dig., µg/g	Dig.	Nitrogen	Dig., µg/g	Dig.	N.	Dig., µg/g	Dig.	N.
				A. Liver homogenate					
5.0	.004	.2	—	.042	2.0	—	2.0	98	—
2.5	N.D.	N.D.	6.3	N.D.	0	8.3	1.8	100	85
2.5	.150	7.4	2.0	.03	1.5	5.8	1.8	91	92
1.3 *	.018	1.9	6.2	.01	.9	4.5	.9	97	89
2.5	.024	1.7	2.1	N.D.	0	8.1	1.4	98	90
2.1	.138	10.2	9.8	.01	.7	15.9	1.2	89	75
2.65	.056	3.6	5.3	.015	.09	8.5	1.5	96.5	86
				B. Heart homogenate					
							Fraction S_2		
2.2	.11	8.5	—	.06	3.2	—	1.1	85	—
2.2	.06	4.8	27	.02	1.6	17	1.1	89	50.0
2.0	.12	5.5	29	.05	2.5	19	2.0	92	44.2
2.13	.096	6.3	28	.04	2.4	18	1.4	88.7	47.1

* Liver perfused with isotonic sucrose *in situ* before homogenization.

mentation of the submicroscopic particles of fraction S_1 at 22000 g resulted in the sediment labelled fraction Pw and the supernatant S_2. The heart homogenates were centrifuged in a Spinco horizontal head which gives particularly well defined layers. All of the fractionations were done at a temperature between 0 and 5°C. The nitrogen of the fractions was determined by direct nesslerization(5).

2. *Extraction of Digitoxin for Bio-Assay.* The Nw, Mw2, Pw, and aliquots of the S fractions were diluted to 10 ml. with Tyrode's solution, then shaken with 25 ml of CH_2Cl_2 for 1 hour at room temperature, allowed to chill overnight at 4°C—or shaken in the cold and centrifuged immediately. The aqueous layer was removed and the protein and CH_2Cl_2 layers separated by filtration. The CH_2Cl_2 filtrate was evaporated to dryness; C_2H_5OH (95%) was added and evaporated twice to remove traces of CH_2Cl_2 from the slight residue. The residue was taken up in Tyrode's media, and assayed for its digitoxin content by the embryonic duck heart method(4). In each experiment, a portion of the intact tissue (or homogenate) also was analyzed for its digitoxin content in order to determine what fraction of the total amount of digitoxin in the tissue was present in each fraction isolated. For control purposes, identical analyses both of intracellular components and whole organ fragments of animals not receiving digitoxin were carried out in each experiment. None of these control samples was found to affect the contractions of the embryonic duck heart preparation.

Results. 1. *The Deposition of Digitoxin in the Hepatic Cell.* As Table I shows, digitoxin when administered to the intact rat appears to enter the interior of the cell. Thus the nuclear and mitochondrial fractions contained 0.056 and 0.015 µg of digitoxin per gram of tissue respectively, 5 minutes after injection of the drug. The deposition of digitoxin, however, in these particular fractions of the homogenate was notably low compared to its accumulation (1.5 µg/g) in the aqueous soluble fraction S_1. This amounted to approximately 96% of the total digitoxin found in all of the fractions extracted. The nitrogen assay of these same fractions suggested that the major portion of cellular protein also was present in this same fraction.

2. *The deposition of digitoxin in the cardiac cell.* As in the above findings, only the supernatant fraction S_2 was found to contain a significant amount of digitoxin (Table I, B). Thus this fraction contained approximately 89% of all digitoxin recovered from the four fractions analyzed. The nitrogen analyses of these same fractions are interesting in that they demonstrate that the deposition of digitoxin is not a simple protein adsorption

phenomenon. For, while the digitoxin content of soluble protein fraction S_2 is 35 times as great as that of mitochondrial fraction Mw2, the nitrogen content of S_2 is less than 3 times that of Mw2.

Discussion. The present results indicate that although digitoxin when administered to the intact animal was capable of entering various cellular components, it appeared to concentrate only in that fraction (S_1 or S_2) of liver or heart containing the soluble constituents of the cell and extracellular fluid. The trace amounts of digitoxin found in the nuclear and mitochondrial fractions suggest that digitoxin probably does not exert its characteristic action either at these intracellular stations, or upon the energy producing enzyme-substrate complexes found therein. Preliminary experiments on the influence of digitoxin on the oxygen uptake of the various fractions appear to confirm this view. Although it is possible that the drug may have a catalytic effect at the levels found, there has been no work reported to substantiate this hypothesis. In fact, all evidence appears to dissociate the glycoside effect from energy producing systems(10). Another possibility, that low recovery is due to very rapid inactivation or destruction of the drug by mitochondrial enzymes during fractionation may be eliminated since digitoxin is not destroyed during 4 hours of incubation under similar conditions. It is possible, however, that the great amount of digitoxin present in fraction S_1 may be stored for later use by the other components of the cell. Studies concerning this possibility are now in progress.

Our interest naturally is centered in the elements of the soluble fraction. According to Hogeboom(6) this fraction is a highly complex mixture containing all the enzymes involved in anaerobic glycolysis, isocitric dehydrogenase, acid and alkaline phosphatase, catalase, cytochrome C and others. Besides these constituents, adenosine deaminase also has been found in this fraction(7). It is of interest that Snellman and Gellotte(8) recently have found that digitoxin is an inhibitor of this enzyme. Finally, in the heart it is this fraction too, which contains the proteins actin and myosin,—two substances which contribute fundamentally to the contractile dynamics of the cardiac cell.

Quite recently, after completion of this report, Harvey and Pieper(9) published a preliminary report about the intracellular distribution of radioactive digitoxin in the guinea pig heart. They report, contrary to our own findings, that the cellular fractions consisting of cellular debris, and of mitochondria, contained the greatest radioactivity and the aqueous soluble fraction, the least. The reason for this discrepancy between their results and ours is not apparent from their precis, but it should be noted that we have analyzed the cellular fractions after injection of the *intact* animal and their fractions were obtained from the isolated heart perfused apparently with a fluid other than blood. Nevertheless, it may be interesting if confirmation of this work could show that accumulation of digitoxin in guinea pig mitochondria, as opposed to the slight deposition in rat mitochondria, provides a basis for the extreme sensitivity of the guinea pig to glycosides, as opposed to the notorious insensitivity of the rat.

Summary. The hearts and livers of animals given intravenous digitoxin were homogenized and separated by centrifugation into nuclear, mitochondrial and homogeneous supernatant fractions. More than 85% of the digitoxin was found in the supernatant fractions of both heart and liver, while the mitochondria of the organs contained 3 and 0.1%.

---

1. Friedman, M., St. George, S., Bine, R. Jr., Byers, S. O., and Bland, C., *Circ.*, 1952, v6, 367.

2. Hogeboom, G. H., Schneider, W. C., and Pallade, G. E., *J. Biol. Chem.*, 1948, v172, 619.

3. Schneider, W. C., and Hogeboom, G. H., *J. Biol. Chem.*, 1950, v183, 123.

4. Friedman M., and Bine, R. Jr., PROC. SOC. EXP. BIOL. AND MED., 1947, v64, 162.

5. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., *Manometric Techniques and Tissue Metabolism*, 2nd Edition, Minneapolis, 1949.

6. Hogeboom, G. W., *Fed. Proc.*, 1951, v10, 640.

7. Schneider, W. C., and Hogeboom, G. H., *J. Biol. Chem.*, 1952, v195, 161.

8. Snellman, O., and Gellotte, G., *Nature*, 1950, v165, 604.

9. Harvey, S. C., and Pieper, G. R., *Fed. Proc.*, 1953, v12, 329.

10. Wollenberger, A., *Pharm. Rev.*, 1949, v1, 311.

Received April 30, 1953. P.S.E.B.M., 1953, v83.

Attempted Enzymatic Phosphorylation of Ribonucleic Acid. (20346)

Alexander L. Dounce and Ernest R. M. Kay.*

From the Department of Biochemistry, University of Rochester School of Medicine and Dentistry, Rochester, N. Y.

It has been suggested by many authors that the nucleic acids play some role in the mechanism of protein synthesis. Spiegelman and Kamen(1) early proposed that the nucleic acids acted as special mechanisms funneling energy into the synthetic process. More recently Pollister(2) made the suggestion that some of the nucleotides in a nucleic acid may be energy rich di- or triphosphates. The possible activity of such proposed phosphorylated compounds in acting as templates for peptide and nucleic acid synthesis has been described in detail by one of the authors(3). The presence of adenylic acid as one of the nucleotides in ribonucleic acid suggested the possibility that myokinase might act as a phosphorylating agent for ribonucleic acid when in the presence of adenosine triphosphate. Experiments have been carried out in this laboratory which have led to the tentative conclusion that the total phosphorus content of intact ribonucleic acid can be increased. This preliminary experimental work may prove to be of some interest in attempting to attack the problem of the role of nucleic acid in protein synthesis, but thus far the experiments are admittedly only suggestive.

Methods. For the experiments undertaken in this study, the ribonucleic acid used was prepared from calf pancreas and calf liver by the use of sodium dodecyl sulfate(4). The ATP used in the experiments was the sodium salt made from Ba_2 ATP previously prepared from rabbit muscle(5). The enzyme *myokinase* was prepared according to the method of Colowick and Kalckar(6) which is stated to yield a 14-fold purified material. The weight of ATP used in the experiments was equal in all cases to the weight of ribonucleic acid in the reaction vessel. Twenty-five mg of the sodium salt of ribonucleic acid were dissolved in 20 ml of water and warmed in a water bath to 30°C. Five ml of ATP solution were added and one ml of the enzyme solution was then added with stirring. The final solution thus obtained was incubated with shaking at 30° for varying periods of time. The pH of the reaction mixture was always between pH 7 and 7.4. At the end of the incubation period the solutions were quickly cooled to 0°C and sufficient 0.01 N HCl was added to lower the pH to 4.5. The solution was then centrifuged in the cold at 14,000 rpm for 30 minutes to remove the contaminating enzyme, together with a very small amount of RNA. The supernatant was decanted into a small beaker containing 1.4 g of NaCl, which was cooled in an ice bath and the pH was raised to 7 by cautious addition of 0.01 N NaOH. The sodium ribonucleate was precipitated by the addition of two volumes of 95% ethanol. The precipitate was washed with ethanol, acetone, and finally was air dried. The material was then dried *in vacuo* over P_2O_5 and was made up in water solution for analysis. An aliquot was digested and was subjected to analysis for total phosphorus (4).

Results. It was found in two experiments that the total ribonucleic acid phosphorus was increased by about 14% over that of the controls when calf liver ribonucleic acid was incubated with ATP in the presence of freshly prepared myokinase. This increase in phosphorus content is perhaps only suggestive of phosphorylation, but when calf pancreas ribonucleic acid was incubated under the same conditions, the total phosphorus was found to have increased above the control in increments ranging from 17 to 48% as shown in Table I. The possibility that inorganic phosphorus or ATP phosphorus was causing this increase was checked by adding excess orthophosphate and ATP to control solutions of calf liver RNA without the enzyme, and reisolating the ribonucleic acid as above. The reisolated RNA

---

* This investigation was supported in part by a research grant from the National Cancer Institute of the National Institutes of Health, Public Health Service.

TABLE I. Phosphorylation of Ribonucleic Acid (Na-PNA Isolated from Calf Pancreas) with Myokinase and ATP.

Exp. No.	Reaction mixture	Time of incubation, min.	% phosphorus in untreated and reisolated PNA samples*
	mg		
1 (a)	25 Na-PNA 25 Na$_4$ATP No enzyme	60	7.5
1 (b)	25 Na-PNA 25 Na$_4$ATP 1 myokinase	60	9.9
	Control sample of Na-PNA, no treatment	—	7.8
2	25 Na-PNA 25 Na$_4$ATP 1 myokinase	15 60	11.2 12.0
3	25 Na-PNA 25 Na$_4$ATP 1 myokinase	60	9.1

* Values represent averages of duplicate colorimetric phosphate determinations.

which had been incubated with excess orthophosphate had an increased total phosphorus content of only 5% over the non-incubated controls. The sample incubated with ATP showed no increase. Thus the reisolation procedure appeared to remove nonnucleic acid phosphorus satisfactorily. When purified desoxyribonucleic acid was substituted for ribonucleic acid in a number of experiments, no phosphorus uptake was observed upon incubation with myokinase and ATP.

It became apparent during these preliminary experiments that the incorporation of extra phosphorus depended on the enzyme preparation used, since with an old enzyme preparation used with calf liver Na-PNA no incorporation of phosphorus was noted over that of the controls, and a variation was noted in the degree of activity of different active enzyme solutions. Since myokinase is thought to be an –SH enzyme and is rapidly inactivated by hydrogen peroxide at neutral pH(6), it is not surprising that an old enzyme preparation should lose activity. The drastic treatment used in preparing myokinase makes it unlikely that other enzymes are present in the preparation which could be affecting our results.

One of the interesting results of this work was the difference in the amount of phosphate added to the ribonucleic acids of two different tissues of the same species. This difference might reflect a difference in the adenylic nucleotide content of these two nucleic acids, but other interpretations are possible. The action of myokinase has been shown by Colowick and Kalckar to effect the following dismutation reaction: 2 adenosine triphosphate $\rightleftharpoons$ adenylic acid + adenosine triphosphate. It is thus not impossible that in the presence of ATP the adenylic nucleotides of ribonucleic acid may be similarly acted upon. It cannot be stated at the present whether the guanylic nucleotide might also be phosphorylated.

The only work known to us of a nature similar to what is here reported is that of Bresler and Nidzyan(7) who found that rat liver homogenates affected a phosphorylation of ribonucleic acid in the presence of ATP. However, the added phosphorus was cleaved by phosphatase, and it is possible that a different type of phosphorylation was occurring.

It should be noted that in our experiments no adenylic deaminase or –SH compound such as cysteine were added. Since myokinase is supposed to be an –SH enzyme and is inhibited by adenylic acid(6), it is possible that better results could be achieved by adding cysteine and adenylic deaminase.

Summary. Tentative results have been obtained which indicate that the phosphorus content of ribonucleic acid can be increased by incubation with myokinase and ATP. An increase in phosphorus content of about 14% above the control values was obtained for calf liver PNA and increases ranging from 17 to 48% were obtained with calf pancreas ribonucleate.

---

1. Spiegelman, K., and Kamen, M., *Science*, 1946, v104, 581.

2. Pollister, A. W., comments in *Chemistry and Physiology of the Nucleus*, Academic Press, New York, 1952, p 159.

3. Dounce, A. L., *Enzymologia*, 1952, v15, 251.

4. Kay, E. R. M., and Dounce, A. L., *J. Am. Chem. Soc.*, in press.

5. Dounce, A. L., Rothstein, A., Beyer, G. T., Meier, R., and Freer, M., *J. Biol. Chem.*, 1948, v174, 361.

6. Colowick, S. P., and Kalckar, H. M., *J. Biol. Chem.*, 1943, v148, 117.
7. Bresler, S. E., and Nidzyan, E. I., *Doklady Akad. Nauk, S.S.S.R.*, 1950, v75, 79; *Chem. Abst.*, 1951, v45, 2994.

Received May 1, 1953. P.S.E.B.M., 1953, v83.

Effect of Mercurials on Thiamine Excretion in Patients with Congestive Heart Failure. (20347)

MICHAEL G. WOHL, CHARLES R. SHUMAN, AND RICHARD TURNER.
(Introduced by Ernest Spiegel.)
From Nutrition Project, Philadelphia General Hospital, and the Department of Medicine, Temple University School of Medicine.

Much interest attaches to the question of thiamine excretion following mercurial diuresis. There are some references to thiamine losses due to diuretics used in the treatment of congestive heart failure; however, this question has not been systematically investigated. The opinion is held generally that the water-soluble vitamins are lost in greater quantities in the urine as the urinary volume increases. Actually, little is known of the mechanisms available in the kidney for regulation of thiamine excretion, or of the manner in which these mechanisms are affected by the action of mercurials upon the renal tubules.

The present study was undertaken to determine the effect of mercurials upon daily output of thiamine in the urine in patients under treatment for congestive heart failure. This work was conducted in conjunction with another study made upon a separate group of patients and control subjects to ascertain the status of thiamine nutrition in patients with chronic heart disease. The statistical analysis of the results obtained in this group of patients appeared to indicate that thiamine deficiency in association with chronic cardiac failure is more than a coincidence(1). The present group of patients was not included in that study because they had received supplemental vit. B complex in conjunction with their cardiac therapy prior to observations recorded in this communication.

Methods and materials. Patients under treatment for congestive heart failure were selected from the medical service of Temple University Hospital. In each instance the patient presented signs and symptoms of cardiac decompensation: orthopnea, edema, marked congestion of the lungs, engorgement of the liver. The causes leading to heart failure were hypertensive heart disease in 5 patients, arteriosclerotic heart disease in 3 patients, mitral stenosis in one patient, and amyloidosis in one patient. The cardiac failure was refractory to treatment with digitalis, mercurial diuretics, salt poor diet and vitamin supplements. Five of the 10 patients studied have succumbed to their disease since initiation of these studies. The food taken by each patient indicated a relatively low protein and caloric intake in most instances. No specific signs or symptoms of thiamine deficiency were elicited, possibly due to the reduced caloric intake as well as vitamin supplementation in the past. Vitamins and mercurials were discontinued for 5 days prior to initiation of the study. The urine was collected on the wards for 24-hour periods. During the period of observation, the patient was given a balanced diet providing approximately 1800-2000 calories and 1.0 to 1.2 mg of thiamine daily. The calculations were based on tables compiled by Bowes and Church(2). Urine collections during the first 3 days for each patient represented the control period. A mercurial diuretic, Thiomerin or Mercuhydrin, 2 cc, was given intramuscularly on the morning of the fourth day. The 24-hour urine collections continued during the day of mercurial injection, the day following, and for one or 2 more days of mercurial injections and days following. The urine was collected for an additional

TABLE I. Mean Urinary Thiamine Excretion and Urine Volumes for 9 Patients on Constant Thiamine Intake during Control Periods and following Administration of Mercurial Diuretics.

	Control day			Day of mercurial			Day following mercurial		
Patients	Thiamine content, µg	Urine, cc	Thiamine, µg/cc	Thiamine, µg	Urine, cc	Thiamine, µg/cc	Thiamine, µg	Urine, cc	Thiamine, µg/cc
C. M.	280.7	500	.561	759.4	1120	.678	208.1	720	.289
E. R.	86.9	1260	.069	282.1	1800	.157	84.6	1800	.047
S. G.	54.1	1095	.049	293.8	1555	.189	517.1	1190	.435
F. B.	247.4	370	.669	366.5	740	.495	903.3	2700	.335
M. M.	156.4	1290	.121	574.8	990	.581	764.4	1250	.612
S. S.	91.1	858	.106	375.8	1467	.256	344.6	1220	.282
V. M.	61.0	700	.087	103.5	1000	.104	185.8	1250	.149
J. M.	24.3	450	.054	543.2	1400	.388	130.0	1450	.090
L. M.	211.8	1102	.192	329.3	2290	.144	636.6	2430	.262
Mean	134.9	847	.212	403.2†	1374†	.332	419.4†	1557*	.278

* Significantly greater than control at $<.02$ probability.
† " " " " " $<.01$ "

control day between the mercurial injections and at the end of the study. The collected urine was measured quantitatively and then a 200 cc aliquot was acidified and kept refrigerated until analyzed for the thiamine content by the method described previously(1). The total thiamine excretion was recorded as well as the amount per cc of urine.

Results. The object of the initial analysis of data (Table I) accumulated was to evaluate the relationship between the absolute thiamine content and the volume of urine excreted by the individual patients. The Spearman coefficient of rank correlation was determined for thiamine content versus the urine volume. Rank correlation appeared sufficient to observe whether or not larger volumes of urine were associated with larger amounts of thiamine in the urine. None of the coefficients differed significantly from zero and hence could have been caused by chance alone even though there is no relationship between ranks in the variables considered. There is no evidence to indicate that high volume of urine is associated with high absolute amounts of thiamine.

	Spearman coefficients of rank correlation		(p) = rho
	Control	Day of mercurial	Day after mercurial
Absolute amt of thiamine vs vol of urine	—.06	—.35	+.20
Thiamine/cc of urine vs vol of urine	—.23	—.55	—.33

In Table I will be found a summary of the urine volume and the mean urinary thiamine excretion data in 9 patients during control and mercurial therapy periods. The 10th patient was not included in the table because of an inadequate number of control determinations. It is clear from the data that the concentration of thiamine in the urine increased from the control day to the day of the mercurial. It was shown previously that, among individuals, higher urine volumes were associated with somewhat lower concentrations of thiamine. Therefore it would appear that the effect of mercurial is to increase the concentration of and absolute amounts of thiamine in the urine.

Discussion. The use of mercurials is generally admitted to be the most effective means of controlling edema in the treatment of congestive heart failure. The effect of these agents upon electrolyte excretion has been adequately studied. However, there have not been sufficient accounts to provide the basis for quantitative treatment of the influence of mercurials upon vitamin excretion. The report of Williams and Bissel(3) dealing with thiamine excretion in various clinical states included 2 patients with congestive heart failure. A pronounced diuresis of thiamine occurred in each patient following mercurial administration after thiamine loading(3). Their observations tended to indicate that the increased thiamine elimination was not due to the increased urine volume. One patient studied in the present series received an intramuscular injection of 10 mg of thiamine after which a mercurial was given. During the next 24 hours, 13.2 mg of thiamine was eliminated. The results obtained in the present series demonstrate that the levels of thiamine excretion following the administration of mercurials are significantly increased over the levels observed on control days.

The urinary thiamine content compared with urine volumes among the individual patients fail to show higher levels of the vitamin with larger volumes of urine. Certain patients with low urine output had a higher level of thiamine excretion than those with greater urine output. These data seem to indicate that the mercurial-induced thiamine excretion is not a function of the increased urine volume. Although the mechanism of mercurial-induced thiamine excretion is obscure at present, the most likely explanation for these results is the depression of renal tubular reabsorption of thiamine.

In support of the impairment of renal tubular mechanisms for thiamine reabsorption by mercurials is the evidence that the vitamin, when administered to patients deficient in vit. B_1, is retained within the cells by conversion to diphosphothiamine(3). The excretion of thiamine after a loading dose administered to B_1-deficient patients is reduced. This deficiency, reflected in the kidneys, would promote an increased rate of conversion of thiamine derived from tubular urine into diphosphothiamine by the phosphorylating systems of the tubular cells. The levels of thiamine excretion would be reduced, thus preventing losses of the vitamin from the body. Thiamine reclaimed by the tubules would be shunted into the blood(3). In normal subjects, thiamine loading doses are rapidly excreted in the urine because of adequate concentration of the vitamin within the tubular cells as well as the other tissues.

The absence of manifestations of thiamine deficiency in patients receiving mercurials over long periods may be related to an inadequate caloric intake. Furthermore, the patients described in this report had received vitamin supplementation which may have replaced the losses produced by the action of the diuretic. The usual finding in chronic heart failure of muscle atrophy and weight loss was present in each of our patients. This has been attributed to the inability to maintain adequate nutrition due to anorexia, impaired absorption and utilization of ingested foods, and the effect of chronic disease upon cellular metabolism. It is apparent that the oral administration of thiamine and other essential food factors is not capable of correcting the nutritional abnormalities found in these patients.

Summary. The effect of mercurials on output of thiamine in the urine was investigated in 9 patients with congestive heart failure. The statistical analysis of the accumulated data indicates that the levels of thiamine excretion following mercurial administration are significantly increased over the levels observed on control days. The data seem to indicate that the mercurial-induced thiamine excretion is not due to the increased diuresis; it is suggested that it may be related to the depressed tubular absorption of the thiamine.

We are indebted to Dr. H. Menduke for statistical treatment of the data, Mr. Martin Brody for technical assistance; Drs. Henry P. Swartz and Carl Alper for advice and supervision of laboratory work, and Dr. Richard A. Kern for permission to use the clinical material.

---

1. Wohl, Michael G., Shuman, Charles R., Turner, Richard, and Fittipoldi, John, J., in press.

2. Bowes, A. DePlanter, and Church, Charles F., *Philadelphia Child Health,* Jan. 1951.

3. Williams, R. H., and Bissel, G. W., *Arch. Int. Med.,* 1944, v73, 203.

Received May 7, 1953. P.S.E.B.M., 1953, v83.

Failure of Somatotrophic Hormone (STH) to Counteract Cortisone Action on Wounds in Mice.* (20348)

David M. Spain and Norman Molomut.

From the Waldemar Medical Research Foundation, Brooklyn, N. Y., and the Department of Laboratories and Research of Westchester County, Valhalla, N. Y.

Cortisone treatment interferes with inflammatory processes as reflected in inhibition of wound healing(1). Selye(2) reports that a pituitary gland fraction, somatotrophic hormone (STH) counteracts enhancing effect on infection produced by cortisone in rats. We therefore proceeded to determine whether STH neutralized the cortisone effect on wound healing in mice. It should be mentioned that in Selye's(2) study, cortisone and STH were used for 14 days, whereas in this study this combination was used for 6 days.

Uniform sized wounds were made under aseptic technic through the cleanly shaven dorsal skin using a special surgical punch, which created two 0.5 cm wounds on each mouse. Groups of 12 mice each were treated: 1) Diluent only; 2) Cortisone 1 mg; 3) STH 1 mg (Armour—Lot No. R285128); 4) Cortisone 1 mg and STH 1 mg. All injections were intramuscular and started 24 hours before wounding and given daily for 5 days thereafter and the mice sacrificed on the 6th day after wounding. Wound areas were observed grossly, excised, formol fixed, sectioned and stained with hematoxylin and eosin for histological study. In addition, all animals were autopsied and the spleen size of the various groups compared.

Findings. There were no significant differences between the cortisone and cortisone with STH treated groups. The wounds in both of these groups showed inhibition of wound healing as compared with the untreated controls and the STH treated mice. The criteria for determining the degree of wound healing were grossly the size of the wound and histologically the amount and character of granulation tissue formation and the degree of re-epithelialization. The latter two groups (STH treated and untreated) did not differ from each other.

Examination of spleen size in the various groups showed that the cortisone exerted its usual effect on reducing the size of the spleen. The addition of STH did not prevent this phenomenon.

Summary. 1. 1 mg daily of somatotrophic hormone does not counteract the effect of 1 mg daily of cortisone on wound healing in mice. 2. 1 mg of somatotrophic hormone daily does not effect wound healing in mice as compared with untreated controls.

1. Spain, D. M., Molomut, N., and Haber, A., *Science*, 1950, v112, 335.
2. Selye, H., *Fed. Proc.*, 1952, v11, 144.

Received May 15, 1953. P.S.E.B.M., 1953, v83.

---

* The authors express gratitude to Armour and Co. for STH and to Merck and Co. for cortone acetate suspension.

Effects of Insulin and Glucose on Utilization of Fructose by Isolated Rat Diaphragm.* (20349)

BRUCE MACKLER AND GEORGE M. GUEST.

From the Children's Hospital Research Foundation and the Department of Pediatrics, University of Cincinnati, Cincinnati, O.

Results of studies by Price *et al.*(1) and Colowick *et al.*(2) suggested that pituitary and adrenal cortical extracts inhibited hexokinase activity in muscle extracts from alloxan-diabetic rats, and that hexokinase activity was restored by addition of insulin. Experimental results were not consistent, since inhibition of hexokinase activity by adrenal cortical extract with restoration of activity by insulin was obtained in only 50% of the experiments performed. In similar experiments reported by Reid *et al.*(3) and Broh-Kahn and Mirsky(4) the findings were again inconstant. Stadie *et al.*(5,6) found no significant effect of insulin or adrenal cortical extract on hexokinase activity in muscle extracts from normal and diabetic rats. Their findings suggested that pituitary extracts impaired the ability of normal rat-diaphragm to combine with insulin, and that the diaphragms from alloxan-diabetic rats did not combine well with insulin. More recently, however, Chernick *et al.*(7,8) have shown that in the alloxan-diabetic rat liver glucose utilization is initially impeded at the hexokinase stage, and Stadie *et al.*(9,10) demonstrated that in rat-diaphragm the acceleration of glycogen synthesis per μg of bound insulin is greater after removal of the pituitary. Results of experiments presented here suggest that in isolated rat-diaphragms insulin increases the utilization of glucose and fructose by accelerating their phosphorylation by hexokinase.

Methods. Adult white rats fasted 24 hours were decapitated and the diaphragms were removed with minimum trauma(11). Each hemidiaphragm was weighed without preliminary washing and placed directly into the medium contained in a small Erlenmeyer flask. After removal of samples for analysis the 2 flasks (a) and (b) were stoppered and incubated with shaking at 37°C. Samples were removed from the flasks for analysis at the termination of the experiments. The composition of the medium in the 2 flasks was identical except that insulin was added to flask (a), 0.5 unit per cc medium. The medium consisted of 0.1 M phosphate (K_2HPO_4-NaH_2PO_4 mixed in proportion to give a pH of 7.6), $MgCl_2$ 0.005 M, $CaCl_2$ 0.001 M, in final volume 4 cc after addition of fructose and/or glucose. Amorphous insulin (W-1302, 19 units/mg) was obtained from the Eli Lilly Co. Fructose was determined by modifications of the methods of Roe(12) and Nelson (13) using filtrates deproteinized with $ZnSO_4$ and $Ba(OH)_2$. The Nelson method(13) was used for the determination of glucose. pH was determined by means of a Cambridge Research model pH meter with glass electrode.

Results and discussion. Table I presents data obtained in paired experiments on rat diaphragm incubated with and without insulin with combinations of fructose and glucose as substrate. Results of preliminary experiments showed that insulin increased the utilization of glucose by rat diaphragm, in accord with work previously published by Stadie and Zapp(11). In a previous report(14) we have shown that both hexokinase and fructokinase are present in rat diaphragm. Fructose is not converted to glucose in rat diaphragm since glucose-6-phosphatase is not present in muscle(15,16). Results of previous work(18,19) have shown that the hexokinase but not the fructokinase pathway of fructose phosphorylation (Fig. 1) is almost completely inhibited by the presence of an equimolar concentration of glucose. In the experiments here presented, with fructose alone as substrate insulin increased the utilization of fructose by rat diaphragm significantly [p $<$.01, determined by the method of analysis of variance(17)]. However, when both fructose and glucose were

---

* Aided in part by a grant from the Nutrition Foundation, Inc.

TABLE I. Results of Experiments on Rat Diaphragm Incubated with and without Insulin with Combinations of Fructose and Glucose as Substrate. 15 experiments.

Insulin in medium	Initial conc., %		Fructose utilized from medium		Final pH
	Fructose	Glucose	mg/g tissue	Time, hr	
+	.2	——	3.22	3	7.41
—	.2	——	2.78	3	7.43
+	.2	——	2.88	3	7.40
—	.2	——	2.19	3	7.44
+	.2	——	3.11	3	7.35
—	.2	——	2.60	3	7.35
+	.2	——	4.73	3	7.43
—	.2	——	2.96	3	7.44
+	.2	——	3.44	3	7.41
—	.2	——	2.47	3	7.42
+	.2	——	3.63	3	7.44
—	.2	——	2.77	3	7.47
+	.1	——	1.31	4	7.39
—	.1	——	0.80	4	7.37
+	.1	——	2.00	4	7.38
—	.1	——	1.05	4	7.39
+	.1	——	2.91	4	7.45
—	.1	——	2.00	4	7.45
+	.1	.1	1.63	4	7.30
—	.1	.1	1.55	4	7.32
+	.1	.1	1.82	4	7.27
—	.1	.1	1.78	4	7.31
+	.1	.1	1.97	4	7.32
—	.1	.1	2.02	4	7.35
+	.1	.1	2.01	4	7.31
—	.1	.1	1.87	4	7.38
+	.1	.1	1.71	4	7.25
—	.1	.1	1.87	4	7.29
+	.1	.1	1.71	4	7.28
—	.1	.1	1.69	4	7.31

in the medium insulin did not affect the utilization of fructose. It appears, therefore, that the metabolism of fructose via the fructokinase system is not affected by insulin, and that insulin accelerates the utilization of glucose and fructose by way of the hexokinase pathway. Since the reactions follow common metabolic pathways after the phosphorylation

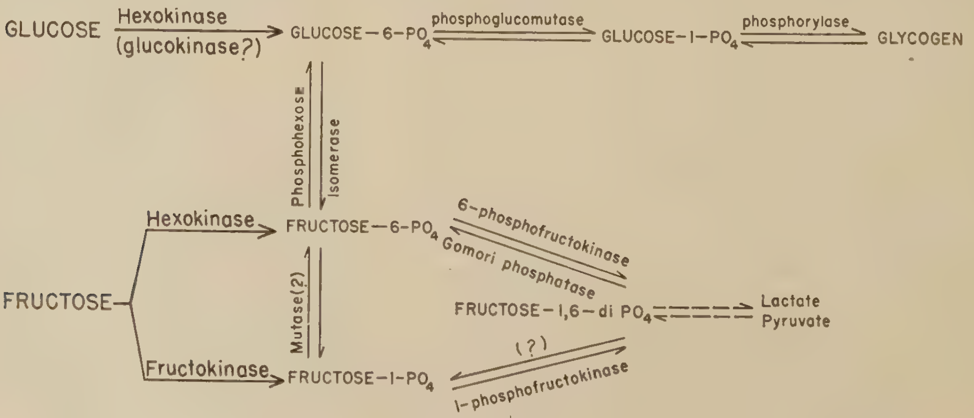


FIG. 1. Pathways for anaerobic metabolism of glucose and fructose in muscle.

of fructose by fructokinase and hexokinase (Fig. 1) it would appear that insulin acts to accelerate the initial phosphorylation of glucose and fructose by hexokinase. It is probable that fructose metabolism in intact animals proceeds almost entirely by way of the fructokinase system since glucose is normally present in tissues *in vivo* in amounts sufficient to inhibit the phosphorylation of fructose by hexokinase. The findings presented in this paper are in agreement with results of experiments *in vivo* by other investigators(7,20-22) suggesting that insulin does not affect the metabolism of fructose in animals and human subjects.

Summary. In isolated rat diaphragm, insulin increased the metabolism of fructose and glucose via the hexokinase system but did not affect the utilization of fructose via the fructokinase pathway. The results suggest that insulin affects the metabolism of glucose and fructose by accelerating their initial phosphorylation by hexokinase.

---

1. Price, W. H., Cori, C. F., and Colowick, S. P., *J. Biol. Chem.*, 1945, v160, 633.
2. Colowick, S. P., Cori, G. T., Slein, M. W., *J. Biol. Chem.*, 1947, v168, 583.
3. Reid, E., Smith, R. H., and Young, F. G., *Biochem. J.*, 1948, v42, XIX.
4. Broh-Kahn, R. H., and Mirsky, I. A., *Science*, 1947, v106, 148.
5. Stadie, W. C., and Haugaard, N., *J. Biol. Chem.*, 1949, v177, 311.
6. Stadie, W. C., Haugaard, N., Hills, A. G., and Marsh, J. B., *Am. J. Med. Sc.*, 1949, v218, 275.
7. Chernick, S. S., and Chaikoff, I. L., *J. Biol. Chem.*, 1951, v188, 389.
8. Chernick, S. S., Chaikoff, I. L., and Abraham, S., *J. Biol. Chem.*, 1951, v193, 793.
9. Stadie, W. C., Haugaard, N., and Marsh, J. B., *J. Biol. Chem.*, 1951, v188, 167.
10. Stadie, W. C., Haugaard, N., and Vaughan, M., *J. Biol. Chem.*, 1953, v200, 745.
11. Stadie, W. C., and Zapp, V. A., Jr., *J. Biol. Chem.*, 1947, v170, 55.
12. Roe, J. H., *J. Biol. Chem.*, 1934, v107, 15.
13. Nelson, N., *J. Biol. Chem.*, 1944, v153, 375.
14. Mackler, B., and Guest, G. M., in press.
15. Cori, G. T., Colowick, S. P., and Cori, C. F., *J. Biol. Chem.*, 1938, v124, 543.
16. Cori, G. T., and Cori, C. F., PROC. SOC. EXP. BIOL. AND MED., 1938, v39, 337.
17. Goulden, C. H., *Methods of Statistical Analysis*, John Wiley and Sons, Inc., New York, 1939.
18. Slein, M. W., Cori, G. T., and Cori, C. F., *J. Biol. Chem.*, 1950, v186, 763.
19. Mackler, B., and Guest, G. M., *Am. J. Physiol.*, in press.
20. Levine, R., and Huddlestun, B., *Fed. Proc.*, 1947, v6, 151.
21. Pletcher, A., Fahrlander, H., and Staub, H., *Helvet. Physiol. et Pharmacol. Acta*, 1951, v9, 46.
22. Miller, M., Drucher, W. R., Owens, J. E., Craig, J. W., and Woodward, H., *J. Clin. Invest.*, 1952, v31, 115.

Received May 18, 1953. P.S.E.B.M., 1953, v83.

Utilization of Sulfur Amino Acids During Healing of Experimental Wounds.* (20350)

MARTIN B. WILLIAMSON AND HERBERT J. FROMM.

From the Department of Biochemistry, School of Medicine and Graduate School, Loyola University, Chicago.

The regeneration of tissue during the healing of wounds has been shown to be markedly accelerated by the addition of methionine to the diet(1-7). More recently, the rate of healing of experimental wounds was shown to be increased to the same extent when equivalent amounts of either cystine or methionine were administered(1). Since the conversion of methionine to cystine is irreversible *in vivo* (9,10), this work may be taken to indicate that cystine is the limiting amino acid in the healing process. The possibility still existed that the sulfur amino acids may not be required *per se*, but rather might serve as a source of sulfur for other compounds required

---

* This work was done under contract with the U. S. Navy, Office of Naval Research.

during healing, as well as their more usual function in protein synthesis.

It has been shown also that there is a small but definite retention of sulfur during healing, even when a negative nitrogen balance is in evidence(1,2,8). Further, the retention of sulfur becomes more significant as the rate of healing increases. Thus, there appears to be a correlation between the sulfur retention and the *healing index*. The *healing index* is a measurable numerical evaluation of a function which is directly proportional to the rate of healing(1,2). The metabolic fate of sulfur and sulfur containing amino acids during the healing of experimental wounds should give an insight into the disposition of the retained sulfur. The utilization of the cystine by the regenerating wound tissue and its relation to the function measured by the *healing index* are considered in this report.

Experimental. The experiments to be described were carried out on two groups of female albino rats (18 animals per group), weighing 170 ± 20 g. The animals were maintained on a basal 0% protein diet for 5 days prior to wounding. After wounding, one group of rats was continued on the 0% protein diet supplemented with 60 mg of DL-alanine per 100 g of diet, while the second group received the 0% protein diet supplemented with 100 mg of DL-methionine per 100 g of diet. The basal diet consisted of 83 g sucrose, 10 g lard, 2 g corn oil, 5 g salt mixture(15), 1 mg thiamine HCl, 1 mg riboflavin, 1 mg pyridoxine HCl, 4 mg calcium pantothenate, 10 mg nicotinic acid amide, 0.5 mg 2-methyl-naphthoquinone, 1550 IU vit. A,† and 210 IU vit. D.† The animals were provided with 7 g of diet per day, which was completely consumed before the next daily feeding period. Distilled water was permitted *ad libitum.* After removing the hair, the wounds were made on the back of the neck of the rats by tracing the outline of a coin (3 cm diameter), and cutting on the marked tracing. The circle of skin was cut down to the layer of the *fascia* and removed with any adhering connective tissue, according to the method described by Paul *et al.*(16). Two days after wounding each rat was injected subcutaneously with 0.85 mg of S^{35}-labeled L-methionine, representing 8.9 x 10^6 counts per minute. One-third of the animals in each group were sacrificed on the 5th, 8th and 12th day after wounding. Samples of regenerating wound tissue, normal unwounded skin, liver and muscle were taken from each rat and hydrolyzed in 6 N HCl. Great care was taken to insure that no normal tissue was included in the sample with the regenerating wound tissue. Normal unwounded skin tissue was obtained from the area near the site of the wound. This tissue was completely freed of hair before analysis. The above tissue samples were analyzed for total sulfur(11), total nitrogen (microkjeldahl), and cysteine + cystine (designated as *cystine*)(12,13). The relatively small amounts of sample available made it necessary to calculate the methionine present in the tissues by difference. Although the methionine-sulfur fractions probably included sulfur from compounds other than methionine, by far the largest part of these fractions of the sulfur comes from the methionine. Besides determining the total S^{35} in the tissue samples, aliquots were partitioned into cysteine + cystine and non-cysteine + cystine fraction by the method of Zittle and O'Dell(14), and the S^{35} determined in the fractions. The latter fraction was designated as the methionine-S^{35} fraction.

Results and discussion. In Table I is shown the S^{35} content of the various tissues which were analyzed in terms of counts per minute per gram of tissue. It should be noted that there is a very marked increase in the S^{35} content of the regenerating wound tissue with time. This increase reflects the accretion of

TABLE I. Effect of Dietary Methionine on Distribution of Methionine-S^{35} in Wounded Rats.

Days after wounding	Counts/min./g of tissue Wound tissue	Skin	Liver	Muscle
	0% protein diet			
5	6065	2327	7986	1660
8	7222	1836	5154	1324
12	9351	1690	4220	924
	0% protein diet + methionine			
5	6272	2705	7475	1252
8	9117	2265	5030	1155
12	11565	1967	3740	865

---

† From *oleum percomorphum.*

TABLE II. Sulfur and Nitrogen Content/g of Wounded Rat Tissue.

Days after wounding	mg							
	Wound tissue		Skin		Liver		Muscle	
	S	N	S	N	S	N	S	N
	0% protein diet							
5	1.9	20.2	2.0	39.9	3.0	34.0	2.8	34.4
8	2.3	22.0	1.7	40.8	2.5	31.5	2.7	33.5
12	2.9	27.8	1.6	42.4	2.3	28.7	2.3	35.6
	0% protein diet + methionine							
5	2.0	22.4	2.1	39.2	2.9	30.5	2.7	34.2
8	2.8	26.3	1.6	42.1	2.5	27.1	2.5	36.1
12	3.6	29.8	1.6	40.2	2.3	26.7	2.4	35.6

TABLE III. Distribution of S^{35} from Methionine between Cystine and Methionine Fractions of Tissue in Wounded Rats.

Days after wounding	Counts/min./g tissue			
	Wound tissue		Skin tissue	
	Cystine*	Methionine	Cystine*	Methionine
	0% protein diet			
5	3536	2417	1465	910
8	4580	3205	1370	730
12	5240	4026	1171	452
	0% protein diet + methionine			
5	3907	2027	1427	1377
8	5177	3600	1085	1225
12	6950	4072	940	1003

* The ''cystine'' fraction includes both cysteine and cystine.

new cells and protein in the wound area. Due to metabolic turnover, the S^{35} content of the other tissues decreased with time. Although some small part of the methionine-S^{35} may have been directly utilized, there can be little doubt that the bulk of the wound tissue S^{35} arose from the degradation products of other tissue proteins.

The total sulfur and nitrogen content of these tissues is tabulated in Table II. The general trend of the total sulfur levels in the tissues parallels that observed for the S^{35}. However, the changes observed are not as marked as in the case of the radio-sulfur. This may account for the previous difficulty in obtaining significant differences in the sulfur content of wound tissues which had been healing for different lengths of time. Particular note should be made of the fact that the sulfur content of the regenerating wound tissues is very much higher than that found in the other tissues analyzed, based on the nitrogen content of the tissues.

The only significant change in the nitrogen content of the various tissues occurred in the case of the wound tissue, although the small decreases observed in the liver may be reflecting the negative nitrogen balance which existed in these animals. The relatively low nitrogen concentrations appearing in the healing wound tissue samples are consonant with previous work which had shown that this type of tissue has a very high water content(16). From the data in Table II, the water content of the wound tissue appears to diminish gradually with the progression of the healing process.

In Table III, the results of the partition of the S^{35} into *cystine* and methionine-S^{35} fractions is shown. It is of special importance to note that so large a proportion of the injected methionine-S^{35} is converted to cysteine- or cystine-S^{35}. In agreement with the data shown in Table I, the sum of the two S^{35} fractions increases with time. However, the concentration of the *cystine*-S^{35} is much greater than is that of the methionine-S^{35}. These results would appear to support the previous report(1) showing that although methionine is required during the healing process, there appears to be greater utilization of cystine.

Table IV shows the results of the analysis of the tissues for *cystine* and methionine. The regenerating wound tissues accumulate *cystine* quite rapidly while the concentration of methionine increases at a more leisurely rate. In the liver and muscle, there was a marked decrease in the methionine concentration while the *cystine* remained essentially unchanged throughout the experiment. This might be taken to indicate that the liver and muscle

TABLE IV. Cystine* and Methionine Content/g Tissue in Wounded Rats.

Days after wounding	Wound tissue Cys (mg)	Wound tissue Meth (mg)	Skin Cys (mg)	Skin Meth (mg)	Liver Cys (mg)	Liver Meth (mg)	Muscle Cys (mg)	Muscle Meth (mg)
				0% protein diet				
5	3.6	4.0	3.0	5.2	4.2	7.9	4.1	7.5
8	5.3	4.2	2.9	5.3	5.0	5.9	4.3	7.4
12	7.3	5.4	2.9	5.4	4.7	4.4	3.9	6.1
				0% protein diet + methionine				
5	4.0	4.1	3.0	4.9	4.5	8.3	4.1	8.3
8	6.7	4.7	2.7	4.9	4.6	6.6	4.1	6.5
12	9.5	5.9	2.8	4.5	5.1	4.7	4.0	6.3

* The "cystine" fraction includes both cysteine and cystine.

methionine sulfur are the principal sources for the wound tissue sulfur. It would also follow that a large portion of the metabolized liver and muscle methionine is converted to cysteine or cystine, which may be utilized by the regenerating wound tissue. This is supported by the fact that, in the animals fed the 0% protein diet, the ratio of *cystine* to methionine excreted was found to be approximately 5:1.

Previously reported analyses, as well as those shown here, indicate that there is usually appreciably more methionine found in most tissues, than there is *cystine*. This, however, is not the case in the regenerating wound tissue. In the wound tissue, the concentration of *cystine* becomes increasingly greater than the level of methionine during the course of healing. This fact might be interpreted to mean that there are two types of proteins being synthesized in the wound area. The first type might be considered to be synthesized relatively slowly and to contain a higher proportion of methionine than *cystine*. The characteristics of this type of protein are probably similar to skin proteins in general. The second type, although not synthesized until at least some of the first type of protein has already been laid down in the wound area, is synthesized more rapidly and contains a higher proportion of *cystine* than methionine. Fractionation studies of the proteins in regenerating wound tissue at various stages of development, and in normal skin tissue, are now under way to test this hypothesis.

The rates of healing of wounds in rats fed a 0% protein diet and a 0% protein diet supplemented with methionine were determined as previously described(1,2). The results are shown in Fig. 1 to be essentially straight lines of different slopes (*healing indices*). When the *cystine* per gram of wound tissue is plotted against time, straight lines also result (Fig. 1). These lines may be represented by the following equations:

$$C = k_1t + a$$
$$T = k_2t + b$$

where C is the *cystine* concentration in the wound tissues, T, the tensile strength, t, the time in days after wounding, and the other symbols represent constants. It would then follow that: $C = k_3T + c$. If this equation is valid, a straight line should result from a plot of tensile strength against the *cystine* content of the wound tissue. That this is actually the case is shown in Fig. 2. It may then be concluded that the rate of deposition of *cystine* in the wound tissue is proportional to the rate of increase in tensile strength of the wound (healing index). Since the rate of healing is proportional to the *healing index*, it can now be measured by two separate functions, the tensile strength of the wound, and the cystine content of the wound area.

It might be assumed that the measurement of the tensile strength of wound tissue, requires, among other factors, the rupture of bonds between relatively large molecules which are not in solution, but are arranged in some sort of colloid meshwork. It would then seem probable that the *cystine* in the wound tissue is preponderantly, if not completely, associated with protein molecules.

Summary. Wounded rats, injected with L-methionine-S^{35}, accumulate the radio-sulfur in the wound tissue, even when the S^{35} content of the other tissues is decreasing. The largest

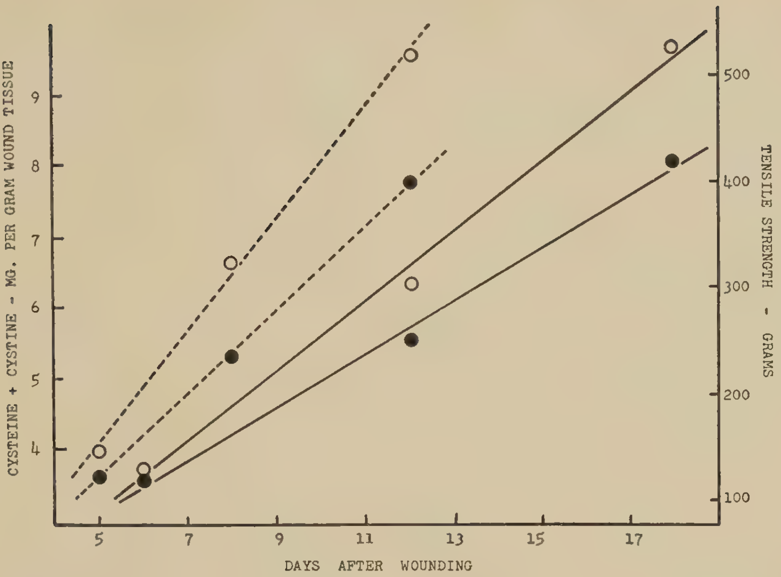


FIG. 1. Tensile strength and cysteine + cystine concentration of healing wounds in rats on a non-protein diet plotted against time. Solid circles, diet supplemented with alanine; open circles, diet supplemented with methionine. Solid lines, tensile strength data; broken lines, cysteine + cystine data. Significance between the mean values of tensile strength is "p" <0.02. Significance between the mean values of the cysteine + cystine is "p" <0.05.

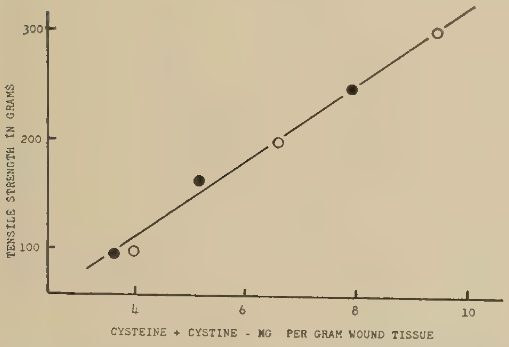


FIG. 2. Tensile strength of healing wound in rats on a non-protein diet plotted against the cysteine + cystine concentration of the regenerating wound tissue. Solid circles, diet supplemented with alanine; open circles, diet supplemented with methionine.

part of the S^{35} in the wound tissue appears as cystine-S^{35}. The rate of deposition of cystine in the regenerating wound tissue is greater than that of methionine. The rate of healing (as measured by the *healing index*) is a function of the cystine content of the wound tissue.

---

1. Williamson, M. B., and Fromm, H. J., PROC. SOC. EXP. BIOL. AND MED., 1952, v80, 623.

2. Williamson, M. B., McCarthy, T. H., and Fromm, H. J., PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 302.

3. ———, *Fed. Proc.*, 1951, v10, 270.

4. Cuthbertson, D. P., *Proc. Nutr. Soc.*, 1946, v4, 185.

5. Localio, S. A., Morgan, M. E., and Hinton, J. W., *Surg. Gyn. Obst.*, 1948, v86, 582.

6. Peters, R. A., *Brit. Med. Bull.*, 1945, v3, 81.

7. Croft, P. B., and Peters, R. A., *Nature*, 1945, v155, 175.

8. Williamson, M. B., and Fromm, H. J., *Fed. Proc.*, 1953, v12, 291.

9. Binkley, F., *J. Biol. Chem.*, 1944, v155, 39.

10. DuVigneaud, V., Kilmer, G. W., Rachele, J. R., and Cohn, M., *J. Biol. Chem.*, 1944, v155, 645.

11. Treon, J. B., and Crutchfield, W. E., *Ind. Eng. Chem.*, 1942, v14, 119.

12. Folin, O., and Marenzi, A. D., *J. Biol. Chem.*, 1929, v83, 109.

13. Block, R. J., and Bolling, D., *The determination of amino acids*, 1948, Burgess Pub. Co., Minneapolis.

14. Zittle, C. A., and O'Dell, R. A., *J. Biol. Chem.*, 1942, v139, 753.

15. Hubbel, R. B., Mendel, L. B., and Wakeman, J., *J. Nutr.*, 1937, v14, 237.

16. Paul, H. E., Paul, M. B., Taylor, J. D., and Marsters, R. W., *Arch. Biochem.*, 1945, v7, 231.

Received May 18, 1953. P.S.E.B.M., 1953, v83.

A Simplified Method for the Preparation of Corticotropin. (20351)

Robert J. Bartholomew.* (Introduced by E. B. Astwood.)

From the Ziskind Research Laboratories, New England Center Hospital, and Department of Medicine, Tufts College Medical School, Boston, Mass.

Previous work in this laboratory has shown that the corticotropic activity of dried pituitary gland can be satisfactorily extracted by glacial acetic acid and that a considerable concentration of the activity can be obtained by adsorption from dilute acetic acid onto cellulose(1) or oxycellulose(2), followed by elution with dilute hydrochloric acid. The specificity of oxycellulose suggested that, through its use, material of satisfactory purity for clinical application might be obtained from very crude extracts, such as those made with 80% acetic acid which has been found to dissolve about half of the dried pituitary tissue.

This present note reports a simplified method for obtaining, from either whole pituitaries of sheep or anterior lobes of pigs, a good yield of a highly active preparation. The actual working time is very small but, as described, is spread over 4 days.

Methods. In a representative experiment 20 g of dried powdered whole pituitary of sheep were stirred to a smooth paste with 90 ml of water, then 360 ml of glacial acetic acid were stirred in to make an even suspension. The mixture was then heated on a steam bath with frequent stirring until the temperature of the mixture reached 70°C. This required about one hour and at the end of this time the mixture had a translucent gelatinous appearance. The mixture was then allowed to cool, and diluted with water to 6 litres in a tall cylinder. Ten g of Hyflo Super Cel were added and the whole stirred mechanically for 10 minutes, and then allowed to stand overnight. The supernatant was then siphoned from the sediment of tissue fragments (which was covered by a compact layer of Super Cel) and transferred to another tall cylinder. Slightly more than 5.6 litres of opalescent supernatant were obtained and filtration was unnecessary. The protein concentration, as estimated by the biuret method(3), was 1.7 mg per ml, representing 51% of the weight of dry gland. To the supernatant was added 9.6 g of oxycellulose (Tennessee Eastman, Lot No. C 1060, 11.0% COOH, 0.13% N) and the mixture stirred mechanically for 24 hours, and then allowed to stand overnight. Before use the oxycellulose was washed on a coarse sintered glass filter with water, normal hydrochloric acid and deci-normal acetic acid. The amount used, 9.6 g, was quite arbitrarily chosen, being approximately equal to the weight of protein in the dilute acid extract. Most of the supernatant was siphoned off and discarded, then the remaining supernatant and the sediment of oxycellulose were transferred to a coarse sintered glass filter, filtered by suction and the residue washed on the filter with 2 or 3 small lots of deci-normal acetic acid. The corticotropin was then eluted from the oxycellulose by 4 washings, each 40 ml, of deci-normal hydrochloric acid, the first and second washings each being left in contact with the adsorbent for about an hour. The protein in the eluate as estimated by the biuret method was 0.90 mg per ml (or 144 mg in the total eluate), which represents 0.77% of the original powdered gland.

Assays were made on the dried pituitary powder and at various stages of the procedure by the ascorbic acid depletion method of Sayers *et al.*(4) as modified in this laboratory to a minimal effective dose method(1). The results in the particular preparation described and those in a similar preparation from anterior lobes of pig are shown in Table I. The values shown for the starting materials are in terms of actual weight of dried gland, the other values are in terms of protein indicated by the biuret estimations. Of the total 6 litres of extract only 5.6 litres (sheep) or 5.4 litres (pig) were used for the succeeding steps. The recoveries shown for the various fractions are not corrected for this.

---

* The Prince Henry Hospital, Sydney, Australia.

TABLE I. Yields in Corticotropic Activity in 2 Experiments Expressed as Minimal Dose to Induce a Fall in Adrenal Ascorbic Acid Concentration between 60 and 100 mg % in Hypophysectomized Rats.

Fraction	Sheep		Pig	
	Wt, g	Minimal effective dose, μg	Wt, g	Minimal effective dose, μg
Acetone-dried whole pituitary	20	10		
Acetone-dried anterior lobes			20	2.5
Extract in N. acetic acid	9.2	5	11.6	2.5
Supernatant after adsorption		>50		>50
Eluate in N/10 hydrochloric acid	0.144	0.1	0.320	0.05

The "direct adsorption method," as described here, was subsequently modified for use on fresh frozen sheep pituitaries. The frozen glands (water content approximately 80%) were rapidly broken into pieces of convenient size, and dropped into a macerator containing 320 ml of glacial acetic acid for each 100 g of frozen glands. The macerator was run at low speed until the lumps of frozen glands had sufficiently disintegrated, then at full speed until thorough maceration had been achieved (about 10 minutes). Heating, dilution and adsorption were then done essentially as in the original procedure, but it was found that centrifuging the dilute acid extract was preferable to sedimentation and that the addition of Super Cel was unnecessary and undesirable.

The method as used on acetone-dried pituitary was briefly described in September, 1951 (5), and was successfully applied by Sydnor and Sayers(6) to the extraction of corticotropin from blood.

Summary. A simple procedure is described whereby a good yield of highly active corticotropin can be obtained by direct adsorption from a crude pituitary extract. The method is equally suitable for pituitaries from sheep and from pigs.

1. Payne, R. W., Raben, M. S., and Astwood, E. B., *J. Biol. Chem.*, 1950, v187, 719.
2. Astwood, E. B., Raben, M. S., Payne, R. W., and Grady, A. B., *J.A.C.S.*, 1951, v73, 2969.
3. Gornall, A. G., Bardawill, C. J., and David, M. M., *J. Biol. Chem.*, 1948, v177, 751.
4. Sayers, M., Sayers, G., and Woodbury, L. A., *Endocrinology*, 1948, v42, 379.
5. Astwood, E. B., Rabin, M. S., and Payne, R. W., *Recent Progress in Hormone Research*, 1952, v7, 1.
6. Sydnor, K. L., and Sayers, G., Proc. Soc. Exp. Biol. and Med., 1952, v79, 432.

Received May 18, 1953. P.S.E.B.M., 1953, v83.

Mast Cell Resistance to Hormonal Influence.* (20352)

James E. Devitt, W. J. Pirozynski, and Peter B. Samuels.†
(Introduced by J. S. L. Browne.)
From the Department of Experimental Surgery, McGill University, Montreal, Canada.

Since their description by Ehrlich in 1879, the tissue mast cells have been a continual source of curiosity for histologists and physiologists. Over the years, these ubiquitous cells have been accused of some 30 different functions. Currently, evidence has been presented that these cells are controlled, or at least influenced, by endocrine glands, *viz.*: the pituitary-adrenal cortex(3,6,7) or pituitary-thyroid(1,2) systems. Only one of the papers (6) that have come to our attention records numerical counts to substantiate the author's opinion. Other workers have presented subjective impressions in lieu of statistically valid counts. The purpose of this study was to investigate tissue mast cell response to hormonal influence, utilizing numerical counts to indicate increased or decreased numbers of cells. The controversial field of tissue mast cell function will be avoided.

---

* Supported by a grant from the Cancer Research Society.

† U. S. Public Health Service Fellow.

Materials and methods. Male piebald rats averaging 225 g weight were used throughout. Tissue mast cells were observed in skeletal muscle from the anterior thigh, mesentery, and heart. A whole mount technic was employed to study the mesentery. Two loops of terminal ileum, each about 20 cm in circumference, were ligated, excised, and mounted with tiny springs on wire frames averaging 8 x 10 cm in dimensions. The mesentery was thereby spread out as a sheet or fan with a circumferential rim of ileum. The specimens of mesentery were fixed in absolute methanol and stained in a 35% alcoholic solution of 0.25% toluidin blue. The hearts and specimens of skeletal muscle were fixed in Carnoy's fluid, cut in serial sections 7 μ thick, and stained with 0.5% toluidin blue in a 30% alcoholic solution. The mast cells of the mesentery were counted in avascular areas using a magnification factor of 150. Forty such fields, 20 from each of 2 specimens, were examined and counted in each animal. A field with a magnification factor of 240 was used in examining the heart and skeletal muscle. In each specimen 375 fields were counted through multiple sections. This larger number of counted fields was necessary because of the sparseness of mast cells in these tissues. Naturally, precautions were taken to avoid overlapping of any of the fields. All counts were made by the same worker, using the same microscope. The morphological appearances of the mast cells were simultaneously noted. Thyroxine (Squibb) and Cortone Acetate (Merck) were injected subcutaneously into the appropriate animals. In the thyroxine studies 19 rats were divided into groups of 9, 5, and 5. Nine received 200γ of thyroxine daily for 7 days. Five rats were starved, being given tap water only for a period of 5 days to rule out any inanition effect of the thyroxine. Five rats were used as controls. In the cortisone series, 24 rats were divided into 3 groups of 8. One of these groups received 10 mg Cortone Acetate daily for 6 days. A second group was given 5 mg for a similar period, and the third was the control group. Loss of weight by all experimental animals was interpreted as indicating the potency of the hormone administered. The mean, standard deviation, and standard error were calculated for each group. The results obtained were analyzed for statistical significance using the *t* test of "Student".

TABLE I. Effect of Thyroxine and Starvation upon Mast Cell Counts in the Rat's Mesentery.*

Treatment	200 γ thyroxine daily	Starved	Controls
Duration	7 days	5 days	—
No. of animals	9	5	5
Mean value	538.6	608.4	485.6
S. D.	± 106.0	± 101.4	± 49.1
S. E.	± 35.3	± 45.5	± 21.9

* Units: No. of cells in 20 fields (× 150).

Results. The experimental data are recorded in Tables I and II.

Statistical evaluation shows that no significant difference exists between the tissue mast cell counts of the thyroxine-treated rats and their corresponding controls. The same holds for the cortisone-treated animals and their control group. Only the starved group of rats shows a statistically significant change in number (an increase) when compared with the associated controls.

A preliminary investigation using desoxycorticosterone acetate failed to reveal any changes in mast cell number.

Typical so-called degenerative changes, *i.e.*, vacuolation, granule conglomeration and irregularity, cellular bursting with granule dispersion, and loss or change in degree of metachromasia, were very frequently observed. However they were seen as frequently in the controls as in the experimental animals.

Discussion. We were unable to effect any alterations of tissue mast cell numbers except in the starved group of animals. Here the increase was probably more apparent than real. It was observed that in this group most of the perivascular fat in the mesentery had been resorbed. This meant that the fields counted probably encroached upon these perivascular mast-cell-rich areas, which in other specimens would have been avoided because the thickness of the fat makes accurate counting impossible.

The figures presented for the cortisone-treated heart specimens are somewhat more impressive when it is realized that 2 of the members of the 5 mg group and one of the 10 mg group gave low counts because the sections were washed out. Thus many mast

TABLE II. Effect of Cortisone upon Tissue Mast Cell Counts in the Mesentery, Skeletal Muscle, and Heart of the Rat.

Tissue	Mesentery*			Heart†			Skeletal muscle†		
Daily dose, mg	10	5	—	10	5	—	10	5	—
No. of animals	8	8	6	8	8	8	8	8	8
Mean value	604.9	568.9	568.8	15.91	14.51	21.44	20.46	16.51	18.39
S. D.	±83.7	±106.7	±78.2	±5.37	±5.59	±7.79	±7.02	±6.31	±3.26
S. E.	±29.6	± 37.7	±27.6	±1.90	±1.98	±2.75	±2.48	±2.23	±1.15

* Units: No. of cells in 20 fields (× 150).
† " " " " " 25 " (× 240).

cells were undoubtedly missed in the examination of these 3 specimens.

Although numerical counting of tissue mast cells is difficult, and not an ideal method of investigating their response to various stimuli, their capricious distribution renders simple estimation of numbers by impression alone worthless. One essential step in making numerical counts is to run a control group with each experimental series. It was found early in this investigation that counts vary significantly from batch to batch of rats, apparently because of age and weight differences. We could attribute none of the "degenerative changes" to treatment the rat had received. It was our impression that these appearances were a function of the fixation-staining-mounting technic. It is, therefore, our opinion that tissue mast cell response can only be adequately measured by actual numerical counts evaluated statistically.

Using such a criterion we were unable to find any suggestion of mast cell susceptibility to hormonal influence. These observations oppose those of Asboe-Hansen(3), Cavallero and Braccini(6), Bloom(5), and Stuart(7), who have reported a decrease in the number of mast cells following cortisone treatment. Baker *et al.*(4) found no morphological or real numerical alteration in the mast cells of the thymus after ACTH therapy, although no counts were presented. Asboe-Hansen(2) has reported differences in mast cell numbers and appearance in patients suffering from myxoedema and thyrotoxicosis. Arvy and Gabe(1) claimed to observe an increase of the mast cells in all tissues following thiourea administration. No counts were offered in either of these articles. We were unable to demonstrate any effect on mast cell numbers following thyroxine treatment.

Summary and conclusions. The effect of cortisone and thyroxine and starvation on the numbers of tissue mast cells in the rat mesentery, heart, and skeletal muscle was investigated by making numerical counts of the cells. A whole mount technic was employed for the mesentery, while other tissues were prepared by the usual histological routine. All were stained with an alcoholic solution of aqueous toluidin blue. No significant changes in mast cell numbers were effected by either cortisone or thyroxine. Starvation produced a statistically significant increase. The alleged degenerative types of mast cells were seen as frequently in controls as in treated rats. This group of experiments therefore has failed to provide any evidence supporting the contention that tissue mast cells are affected by thyroxine or cortisone.

The authors wish to thank Mrs. Elaine Solkin for technical assistance.

---

1. Arvy, L., and Gabe, M., *Experientia,* 1950, v6, 23.

2. Asboe-Hansen, G., *Acta Derm.-Vener.,* 1950, v30, 221.

3. ———, Proc. Soc. Exp. Biol. and Med., 1952, v80, 677.

4. Baker, B. L., Ingle, D. J., and Li, C. H., *Am. J. Anat.,* 1951, v88, 313.

5. Bloom, F., Proc. Soc. Exp. Biol. and Med., 1952, v79, 651.

6. Cavallero, C., and Braccini, C., Proc. Soc. Exp. Biol. and Med., 1952, v78, 141.

7. Stuart, E. G., *Anat. Rec.,* 1951, v109, 351.

Received May 21, 1953. P.S.E.B.M., 1953, v83.

Eosinopenia During Last Third of Pregnancy and After Delivery in Several Stocks of Mice.* (20353)

FRANZ HALBERG AND FRED BOCK. (Introduced by M. B. Visscher)

From the Department of Physiology, University of Minnesota.

In the course of other work it was noted that the eosinophil count of mice in an advanced stage of pregnancy, or within a day or two after delivery, was lower than that of non-pregnant females of the same stock. The data presented in this report appear to verify this impression.

Materials and methods. Pregnant and non-pregnant mice from the breeding colony of the Division of Cancer Biology at the University of Minnesota were used for study. The stocks employed have been described(1). Mice for which counts were obtained on the day of delivery or on the following day constitute Group I (post partum). The other mice studied had not delivered within 3 weeks prior to sampling. Some of them (Group II) were over 2 weeks pregnant at the time of sampling and delivered their young within the following 7 days. Other mice, which had not shown a marked increase in weight and which did not deliver young during the 32 days following sampling constitute Group III (the "control" group). It is possible that mice which had abortions were included in this latter group. The mice constituting Group III were selected to match in age (± 10 days) the mice of the experimental groups, which were from 3 to 10 months of age. The animals were individually housed for 36 to 96 hours prior to sampling. The cages were kept in a room in which activities necessary for the maintenance of other mice continued without change. Room temperature varied between 73 and 85°F. The room was illuminated during the day and dark at night. Purina Fox Chow and tap water were available to the mice, *ad libitum,* from weaning and throughout this investigation. Samples of blood were obtained during specified periods of the day and the eosinophil counts were recorded with notation of the time. The procedures employed for eosinophil counts have been described(1).

Results. It appears from Fig. 1 that eosinopenia is characteristic of the first 2 days after the delivery of the young, in the stocks of mice examined. Table I indicates that on the average the eosinophil level of mice pregnant 2 weeks and more is lower than that of non-pregnant mice of the same stock. Examination of the 2 sections of Table I also reveals that eosinopenia during the third week of pregnancy is observable in data from samples collected at night as well as in data from samples collected during the day.

Discussion. It can be seen from Table I that the differences observed in mean eosinophil level between pregnant and non-pregnant mice of the Ax and Zb stock are significant below the 5% level. Comparable data describing mice of the A stock and the Z stock show the same trend, even though the differences observed are not significant statistically.

A mice differ from Ax mice and Z mice differ from Zb mice by the presence of the mammary tumor milk agent. The presence of this agent does not appear to influence the characteristic eosinophil level in 6-months-old males of the A and the Z stocks(1). The question whether or not the presence of the agent has an effect on the degree of eosinopenia noted in females of 2 stocks of mice prior to and shortly after the delivery of the young cannot be discussed on the basis of these limited data. If the problem of a possible effect of the milk agent on eosinophil levels during pregnancy should be of interest to other workers, the standardization of the circumstances of study may prove to be imperative. Routine conditions prevailing in a breeding colony do not seem to be satisfactory for the quantitative evaluation of a variable as sensitive to environmental factors as the eosinophil level. For the same reason a comparison of the day and night levels obtained

---

* This investigation was supported by a grant from National Institutes of Health, Public Health Service.

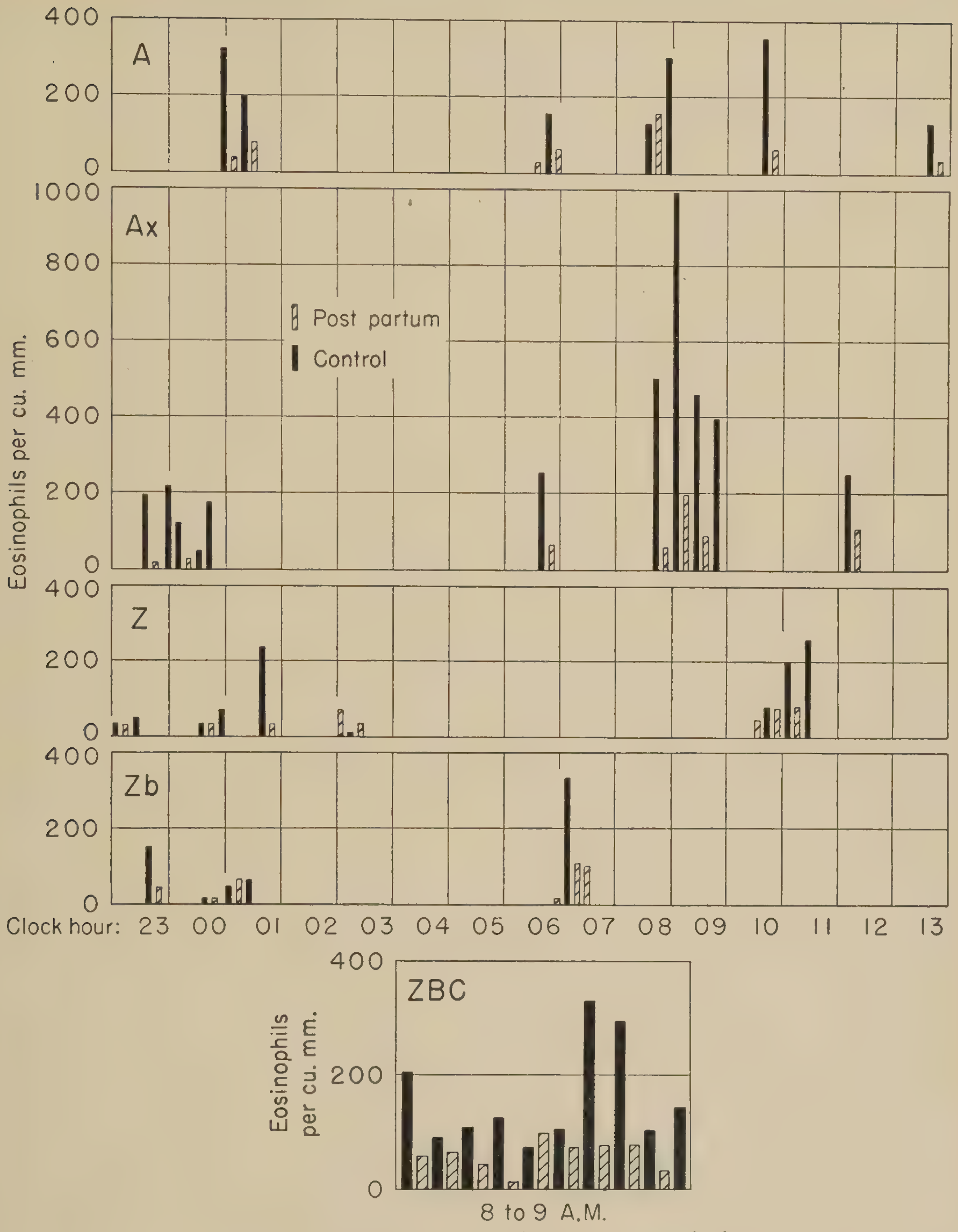


FIG. 1. Post partum eosinopenia in several stocks of mice.

for the stocks studied does not appear to be justifiable.

The observation of eosinopenia post partum in mice is in keeping with an earlier finding on the human. Davis and Hulit reported a marked decrease in number of eosinophils during labor as well as the persistence of low eosinophil levels through the first day post

TABLE I. Eosinopenia during the Last Third of Pregnancy in the Mice Investigated.

Stock	Type	No. of mice	Mean No. of eosinophils	Difference	t	P_t
Section I. Samples obtained from 08:30 to 13:00						
Zb	NP	4	133	73	2.598	.026
	P	8	60			
Z	NP	8	121	47	1.289	.217
	P	9	74			
Ax	NP	5	518	428	2.966	.021
	P	4	90			
A	NP	4	164	32	.923	.375
	P	10	132			
Section II. Samples obtained from 22:00 to 02:30						
Zb	NP	7	66	51	2.481	.029
	P	7	15			
Z	NP	4	80	42	1.023	.333
	P	7	38			
Ax	NP	9	212	111	2.837	.011
	P	10	101			
A	NP	6	174	53	2.966	.260
	P	8	121			

NP = nonpregnant; P = pregnant.

partum(2).

Summary. Eosinopenia was observed during the last third of pregnancy in several stocks of mice. The mean eosinophil level of females of the Ax and the Zb stock, pregnant 2 weeks and more, was less than half of that noted in non-pregnant mice. Eosinopenia was also noted during the 48 hours following the delivery of the young.

The authors are indebted to Dr. Maurice B. Visscher for advice, to Dr. John J. Bittner for the mice employed and to Miss Ruth O. Vrudny for valuable assistance.

---

1. Halberg, F., Bittner, J. J., and Visscher, M. B., *Blood*, 1951, v6, 832.

2. Davis, M. E., and Hulit, B. E., *J. Clin. Endocrinol.*, 1949, v9, 714.

Received May 22, 1953. P.S.E.B.M., 1953, v83.

Serial Propagation *in vitro* of Agents Producing Inclusion Bodies Derived from Varicella and Herpes Zoster.* (20354)

THOMAS H. WELLER. (Introduced by J. F. Enders.)
(With the technical assistance of Helen M. Witton.)

From the Research Division of Infectious Diseases, Children's Medical Center, Boston and the Department of Tropical Public Health of Harvard School of Public Health.

It is generally accepted that serial propagation in the laboratory of the agents of varicella and of herpes zoster has not heretofore been accomplished. Certain of the earlier published reports regarding their serial propagation have more recently been attributed to possible confusion with viruses pathogenic for lower animals. However, morphological evidence has suggested that a single passage of the agents of varicella and herpes zoster has been achieved. Thus Rivers(1,2) observed focal lesions with intranuclear inclusions in the monkey testicle following the local inoculation of varicella vesicle fluid. Goodpasture and Anderson(3) grafted human skin on the

---

* Aided by a grant from the National Foundation for Infantile Paralysis.

TABLE I. Isolation of Cytopathogenic Agents in Tissue Cultures Inoculated with Varicella Vesicle Fluid.

Patient	Age	Day illness vesicles aspirated*	Vesicle fluid frozen before use	Isolation in roller tubes†	Day cult. C.P.‡ first observed
McE.	23	4	24 mo	3/3	8
Wel.	4	2	22 ”	3/4	8
Rud.	7	2	9 ”	3/4	6
And.	8	1	1 ”	2/4	6
Dru.	15	3	2 wk	3/4	7
Pat.	4	1	1 ”	1/4	13

* Dated from appearance of vesicles.
† No. cultures showing foci/No. inoculated.
‡ Cytopathic changes.

chorioallantoic membrane of the chick embryo. Although negative results were obtained following inoculation of varicella vesicle fluid, in one experiment histological examination of grafts following inoculation with fluid from herpes zoster lesions revealed intranuclear inclusions. Using essentially the same technic, Blank, Coriell and Scott(4) likewise in one instance demonstrated inclusion bodies after inoculation of zoster material.

In 1948 we began an investigation of the potentialities of suspended cell cultures of human tissues as a medium for the isolation of the causative agent of varicella. Eosinophilic intranuclear inclusions were demonstrated on examination of tissue fragments removed from the cultures at intervals after the introduction of varicella vesicle fluid(5). Efforts to maintain the responsible agent on serial passage in this type of culture were unsuccessful. More recently roller tube cultures of human tissues have been applied to the same objective. The results so far obtained are here reported in a preliminary manner. As will be shown it has been possible to isolate and maintain in serial passage cytopathogenic agents apparently derived from specimens of varicella vesicle fluid as well as others apparently obtained from herpes zoster vesicle fluid.

Materials and methods. Vesicle fluid specimens were collected and stored as previously described(5). Roller tube cultures were prepared as in our studies on poliomyelitis(6) utilizing either human embryonic skin-muscle tissue or foreskin tissue obtained from boys between the ages of 3 months and 3 years. In the present experiments the nutrient fluid for the cultures consisted of bovine amniotic fluid (90%), beef embryo extract (5%), horse serum (5%), antibiotics, soybean trypsin inhibitor and phenol red as recently described (6,7). Changes of the nutrient fluid were made at 3- or 4-day intervals. In certain experiments for histologic examination tissues were grown on coverslips coated with chicken plasma that were placed in roller tubes; these preparations were fixed with Zenkers-acetic acid and stained with hematoxylin and eosin.

Experimental. 1) *Studies with varicella vesicle fluid.* Vesicle fluids derived from 11 cases of varicella were inoculated individually, usually in the form of 0.1 ml aliquots of a suspension in milk, into groups of roller cultures of human embryonic skin-muscle tissue. The exact concentration of the fluid in milk cannot be stated. In every instance tissue growth was well established at the time of inoculation. As summarized in Table I, in cultures inoculated with fluids from six of these cases, focal cytopathogenic lesions of a characteristic appearance developed which were readily seen on microscopical examination of the living cultures. These lesions usually were first observed from the 6th to 8th day after inoculation. They consisted of small collections of swollen, rounded refractile cells which contrasted sharply with the surrounding fibroblastic or epithelial outgrowth. Those foci of affected cells developing in the sheets of cells of normal appearance increased slowly in size. The cells in the center of such focal areas gradually degenerated over the course of several days, while slow peripheral extension of the lesion continued for days or weeks as contiguous cells became infected. Study of stained coverslip preparations from

TABLE II. Serial Propagation of Two Agents Isolated following Inoculation of Varicella Vesicle Fluid in Cultures of Human Tissue.

			McE. strain		Wel. strain	
Passage	Tissue	Cumulate days in culture	Day C.P.* noted	No. cult. C.P. / No. cult. inoc.	Day C.P. noted	No. cult. C.P. / No. cult. inoc.
1	Emb. S-M†	0	8	3/3	8	3/4
2	”	9	3	3/3	4	2/3
3	Foreskin	20	10	1/2	5	2/2
4	”	34	5	8/9	5	10/16
5	”	45	4	3/4	3	3/3
6	Emb. S-M	58	3	15/15	3	15/15
7	”	67	3	9/10	3	10/10
8	”	81	3	8/8	3	8/8
9	”	94	3	6/6	3	6/6
10	Foreskin	124	4	5/6	4	6/6

* C.P. = cytopathic changes.

† Emb. S-M = human embryonic skin and muscle tissue.

roller tube cultures inoculated with vesicle fluid material has shown that the changes in cellular morphology are characteristically associated with the presence of inclusion bodies. At the margin of a focus occasional cells may be observed that show no gross morphological changes, yet contain small granular intranuclear inclusions. Rounded and swollen cells almost invariably possess intranuclear eosinophilic inclusions. Occasionally the larger swollen cells are multinucleate, with each nucleus containing an inclusion; such cells resemble the multinucleate giant cells described by various workers in the lesions of varicella, herpes zoster and herpes simplex(8). Focal lesions developing in a pre-existing loose network of cells progress irregularly, but manifest similar changes. The morphological changes observed in the cultures will be reported in more detail in a future communication.

All six of the agents isolated following the inoculation of varicella fluids have been propagated serially in tissue culture as indicated by the successive development in subcultures of focal areas of cellular enlargement and degeneration. Two of the strains (McE. and Wel.) have now been maintained for 10 passages as summarized in Table II. The inoculum employed to initiate the third and succeeding passages in these experiments consisted of 0.1 ml of a suspension of coarsely ground tissue removed from the preceding set of cultures. On one occasion, the infected tissue suspension was frozen in the CO_2 box prior to use; otherwise, the inoculum was prepared on the day it was employed. Fig. 1 to 3 depict representative cytopathic changes observed during the serial propagation of the McE. and Wel. agents. In contrast to the results obtained with tissue suspensions, attempts to establish passages with inocula consisting of centrifuged fluids removed from the infected cultures have so far been unsuccessful.

Studies designed to elucidate the nature and etiological relationship of these cytopathogenic agents thus isolated to varicella are in progress. In one experiment, vesicle fluid material of known infectivity for cultures did not produce cytopathic changes when inoculated following heating at 60°C for 30 minutes. In none of more than 70 control cultures maintained during the passage experiments have focal lesions been observed; these tubes routinely received inocula consisting of a suspension of tissue derived from control cultures of the preceding passage. Tissue suspensions infective for cultures have been without obvious effect when inoculated into suckling mice or into the developing hens egg by various routes. Attempts at *in vitro* neutralization of the cytopathogenic effect with convalescent serum from cases of varicella have so far been unsuccessful. It is possible that the close association of the infective agents with cell constituents may be involved in this apparent lack of neutralizing effect. In certain preliminary experiments in which culture fluids harvested from tubes showing cytopathic changes have been employed as antigen in

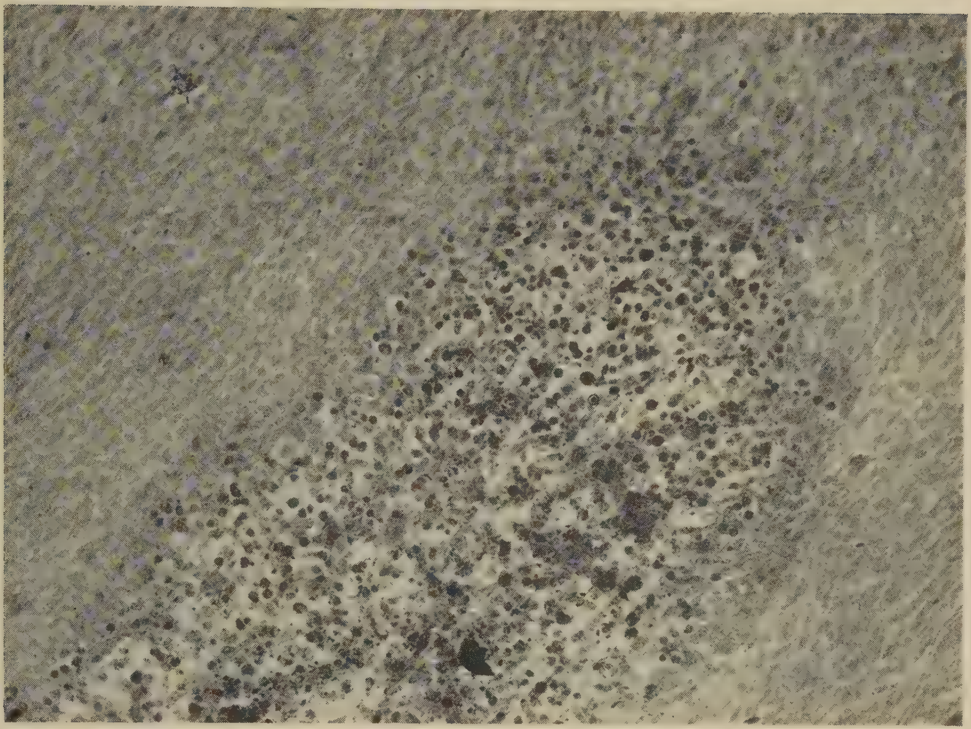


FIG. 1. Varicella. McE. strain; 3rd tissue culture passage. Focal lesion in foreskin culture; 7th day after inoculation. (H & E × 100).

complement fixation tests on paired serum specimens obtained from cases of varicella, a rise in antibody titer has been observed. The specificity of this reaction is under investigation.

2) *Studies with herpes zoster vesicle fluid.* Similar focal cytopathic changes have been observed in cultures inoculated with material obtained on the day of appearance of vesicles from an 80-year-old (Sto.) with thoracic herpes zoster† and with fluid collected from a 30-year-old woman (Pie.) with involvement of the ophthalmic branch of the trigeminal nerve. The *Sto.* inoculum had been stored in the frozen state for 21 months prior to use, while the *Pie.* fluids were inoculated on the day of collection. Serial propagation in tissue culture of these two agents has been accomplished, as summarized in Table III, again with inocula consisting of ground tissue suspensions. (These manipulations have been performed in a separate room from that in which the varicella agents have been maintained.) The cytopathogenic effect observed in the cultures closely resembles that obtained following the introduction of varicella vesicle fluid. Examination of stained preparations from the second tissue culture passage of the *Sto.* strain has also revealed that the rounded swollen cells composing the focal lesions contain intranuclear inclusions (Fig. 4). Tissue suspensions prepared from cultures of the *Sto.* and *Pie.* agents, that were successfully employed to initiate culture passages, have produced no overt symptoms in newborn mice when inoculated by various routes.

Discussion. Inoculation of roller tube cultures of human tissues with materials derived from the eruptive lesions of varicella and of herpes zoster has revealed the presence of cytopathogenic agents capable of producing intranuclear inclusions. These, subsequently, have been maintained in serial passage. A peculiarity of their behavior in the culture system employed has been the apparent failure of infectious material to appear in the fluid phase. The focal lesions appear to in-

---

† Collected by Dr. F. C. Robbins.

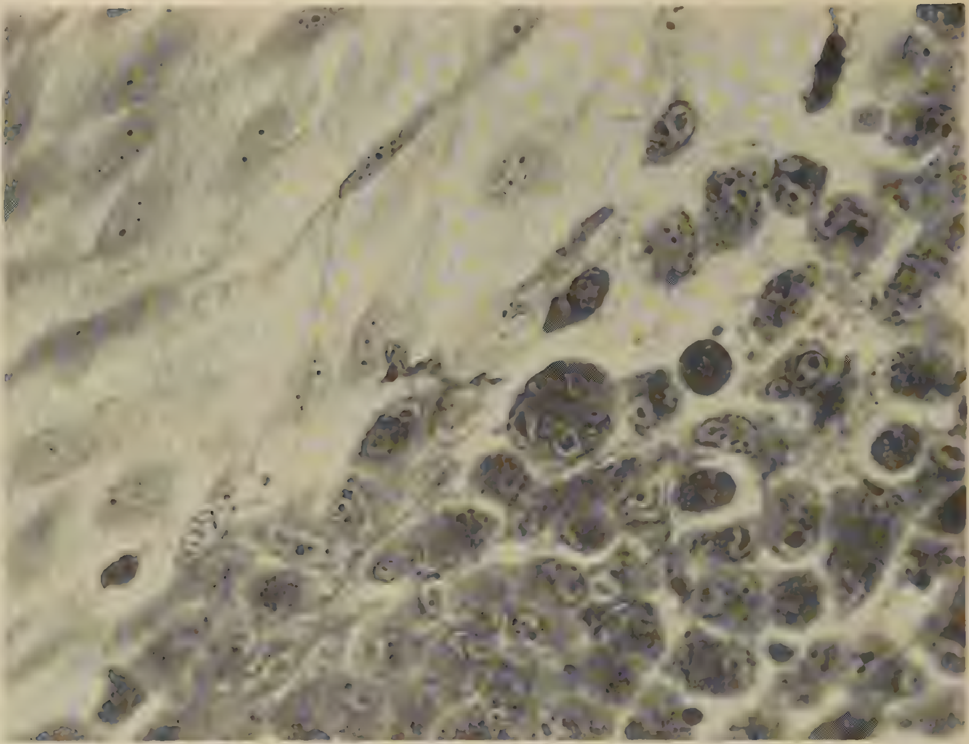


FIG. 2. Varicella. Wel. strain; original passage in human embryonic skin-muscle tissue. Edge of focal lesion on 6th day after inoculation of vesicle fluid. Note intranuclear inclusions and multinucleate cells. (H & E × 550).

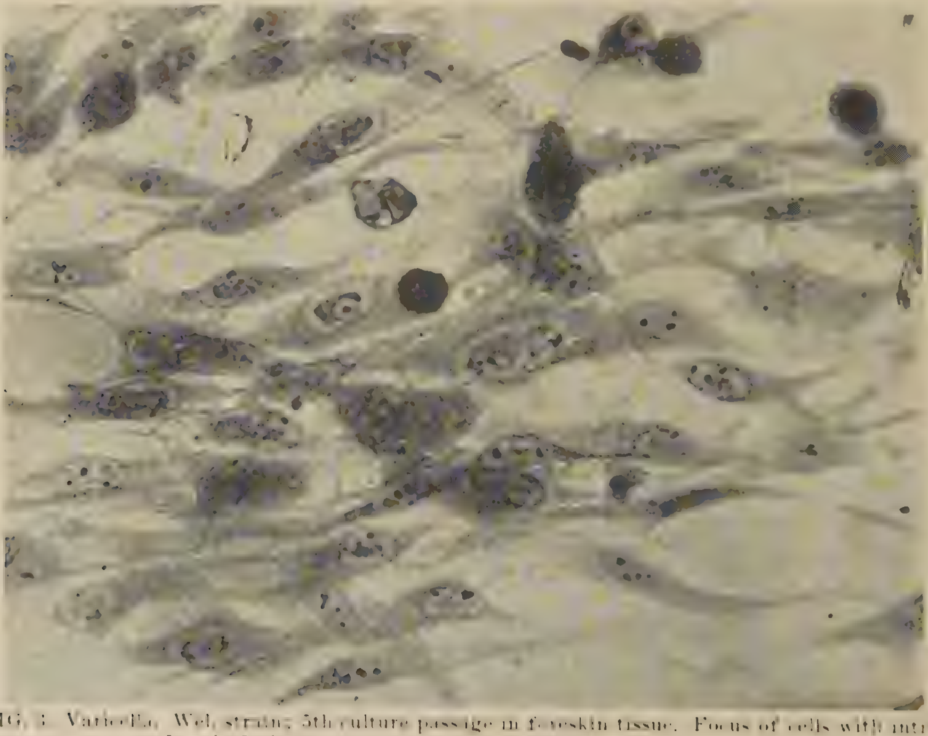


FIG. 3. Varicella. Wel. strain; 5th culture passage in foreskin tissue. Focus of cells with intranuclear inclusions on 6th day after inoculation. (H & E × 550).

TABLE III. Serial Propagation of Cytopathogenic Agents Isolated following Inoculation with Herpes Zoster Vesicle Fluid.

	Sto. strain				Pie. strain			
Passage	Tissue	Cumulate days in culture	Day C.P.* noted	No. cult. C.P./No. inoc.	Tissue	Cumulate days in culture	Day C.P. noted	No. cult. C.P./No. inoc.
1	Emb. S-M	0	6	2/2	Foreskin	0	5	3/6
2	"	15	5	10/10	"	14	3	6/6
3	Foreskin	36	3	6/6	Emb. S-M	44	5	2/3
4	"	66	3	5/6				

* C.P. = cytopathic changes.

crease in size by infection of immediately adjacent cells. On prolonged cultivation, however, an increase in the number of focal lesions occurs. In one experiment in which infected cultures were maintained for 8 weeks approximately 90% of the proliferating tissue became involved. Even so, extension of the lesions was continuing at the end of this period. The significance of these observations is being investigated. It is at present clear, however, that in the utilization of tissue culture technics for the isolation of unknown agents, consideration must be given to passage of tissue suspensions, as well as to the passage of fluid inocula.

No evidence has been obtained that the agents isolated are not those responsible for varicella and herpes zoster. Yet no definitive statement can now be made regarding their nature, their etiological relationships, or the interesting question of the possible identity of the agents of herpes zoster and varicella. It is to be noted that the virus of herpes simplex when propagated in tissue cultures of the type here employed manifests an early focal tendency to induce lesions but then rapidly

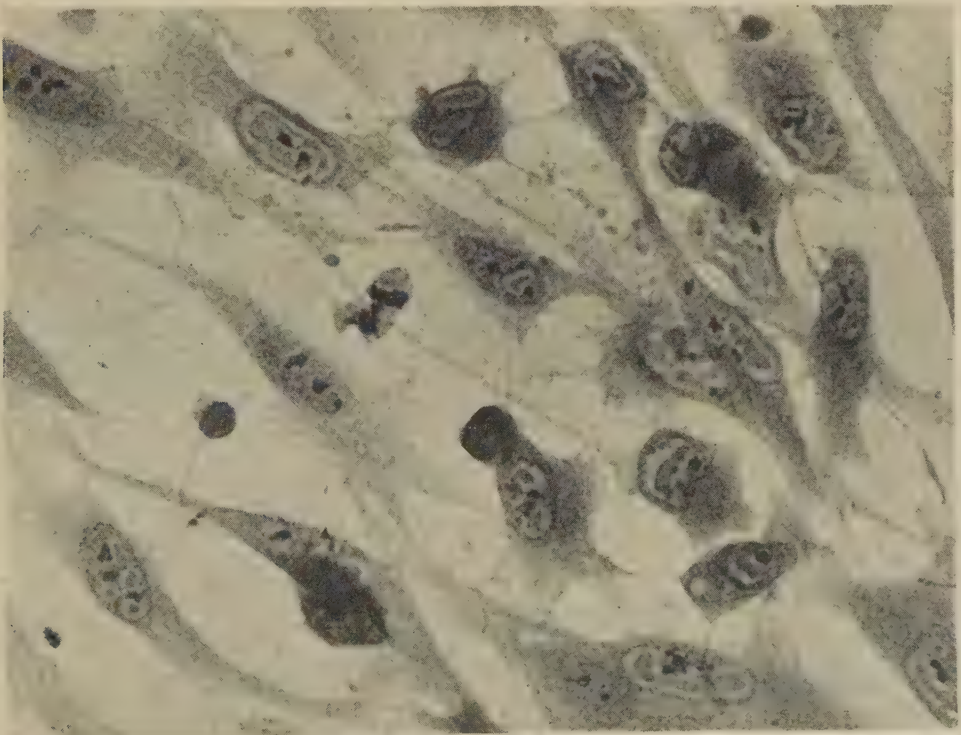


FIG. 4. Herpes zoster. Sto. strain; 2nd culture passage in human embryonic skin-muscle tissue. Edge of focal lesion with intranuclear inclusions on 12th day after inoculation. (H & E × 825).

brings about a widespread infection of the cell population resulting in an appearance quite dissimilar to that here described(9). The failure of these agents to produce obvious infection of suckling mice, in view of the high degree of susceptibility of this animal to the virus of herpes simplex(10), also indicates that they are not to be identified with the latter. We have no indication that a Rickettsia-like agent of the type described by Sprunt and Hirst(11) was present in our cultures.

Summary. The inoculation of roller tube tissue cultures of human tissues with vesicle fluid derived from patients with varicella has resulted in the isolation of six cytopathogenic agents. Two of the strains have been maintained for 10 tissue culture passages by employing tissue suspensions as the passage material. Histologically the lesions produced consist of focal accumulations of cells which become swollen, and then degenerate; characteristically, such cells contain intranuclear inclusion bodies. From the eruptive lesions of two cases of herpes zoster, cytopathogenic agents of a similar nature have been isolated and have been propagated serially in cultures of human tissue.

1. Rivers, T. M., *J. Exp. Med.*, 1926, v43, 275.
2. ———, *J. Exp. Med.*, 1927, v45, 961.
3. Goodpasture, E. W., and Anderson, K., *Am. J. Path.*, 1944, v20, 447.
4. Blank, H., Coriell, L. L., and Scott, T. F. M., PROC. SOC. EXP. BIOL. AND MED., 1948, v69, 341.
5. Weller, T. H., and Stoddard, M. B., *J. Immunol.*, 1952, v68, 311.
6. Robbins, F. C., Weller, T. H., and Enders, J. F., *J. Immunol.*, 1952, v69, 673.
7. Enders, J. F., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 100.
8. Blank, H., Burgoon, C. F., Baldridge, G. D., McCarthy, P. L., and Urbach, F., *J. A. M. A.*, 1951, v146, 1410.
9. Enders, J. F., and Robbins, F. C., unpublished observations.
10. Kilbourne, E. D., and Horsfall, F. L., *J. Immunol.*, 1951, v67, 321.
11. Sprunt, K., and Hirst, G. K., PROC. SOC. EXP. BIOL. AND MED., 1952, v80, 178.

Received May 25, 1953. P.S.E.B.M., 1953, v83.

Antagonism of Toxicity of Vanadium by Ethylenediamine Tetra Acetic Acid in Mice. (20355)

WALTER G. MITCHELL. (Introduced by H. E. Stokinger.)

From the Toxicology Section, Occupational Health Field Hdqrs. U.S.P.H.S., Cincinnati, O.

It has been shown by a number of workers (1-3) that ethylenediamine tetra acetic acid (EDTA) forms complexes with many metals. This principle has been applied, to a very limited extent, to experimental lead and cobalt poisoning in animals(4-6).

Only occasional references to antidotes for vanadium are to be found in the literature. Priestly(7) found that potassium ferrocyanide and substances containing tannin were effective antidotes for vanadium. BAL on the other hand, according to Sjoberg(8), was not only ineffective in reducing vanadium toxicity by inhalation in rabbits, but actually worsened the effect.

In view of the peculiarly strong metal-complexing potentialities and low toxicity of EDTA, and the possible need for an effective antidote in acute cases of vanadium poisoning, the prophylactic value of EDTA was tested in vanadium-poisoned mice. This study constitutes a part of a larger program on vanadium toxicology currently in progress in these laboratories.

Materials and methods. The calcium disodium salt of EDTA* was used for this study, because it has been shown(9) that the sodium salts of this compound injected into an animal

---

* This salt was prepared by combining millimolecularly equivalent amounts of Na_2EDTA (Eastman) and $CaCl_2$. Grateful acknowledgement is made of the generous contribution of a solution of $CaNa_2EDTA$ by the Bersworth Chemical Co., used for part of this study.

TABLE I. Dosage-Mortality Data of Vanadium as $NaVO_3 \cdot H_2O$ in Mice.

mg/kg $NaVO_3 \cdot H_2O$	mg/kg as vanadium	No. of animal deaths to No. used	Mortality, %
20	7.3	3/20	15
25	9.1	7/20	35
30	10.9	14/20	70
40	14.6	18/20	90
50	18.3	19/20	95

in sufficient amount will combine with calcium, causing hypocalcemic tetany and death. The calcium disodium salt reduces this tendency to the extent that the toxicity is decreased 10-fold or more. *Colorless solutions* of hydrated sodium metavanadate ($NaVO_3 \cdot H_2O$) were prepared in physiologic saline at a concentration of 4 mg/ml with a resultant pH of 6.8. Male mice of the Webster strain were used.[†] Each mouse was weighed and given an intraperitoneal dose of EDTA and vanadium according to body weight. EDTA was first administered into the left side of the peritoneal cavity, followed by an injection of sodium metavanadate fifteen minutes later into the right side. Evidence for the validity of this procedure is presented below. An observation period of one week was used in all tests.

Results. Table I shows the dosage mortality data obtained from intraperitoneally administered vanadium, as $NaVO_3 \cdot H_2O$, at 5 dosage levels. The LD_{50} for 20-29 g white mice was 27.2 mg/kg $NaVO_3 \cdot H_2O$ (9.9 mg V/kg), obtained by plotting the data in Table I.

The validity of these data was checked at the LD_{70} and LD_{90} dosage levels.[‡] In these tests, the mortality at the 30 mg/kg level of $NaVO_3 \cdot H_2O$ was 14/20 or 70%; at 40 mg/kg, the ratio was 19/20 or 95%.

Mice pretreated with $CaNa_2EDTA$, 1500 mg/kg, intraperitoneally and injected with 14.6 mg/kg and 18.3 mg/kg vanadium as $NaVO_3 \cdot H_2O$ fifteen minutes later, gave the results shown in column 3 of Table II. At the LD_{95} (18.3 mg V/kg) there was 45% protection; at the $LD_{92.5}$ (14.6 mg V/kg) the protection was 77.5%. The administration of 1500 mg/kg $CaNa_2EDTA$ intraperitoneally in a group of 20 mice not injected with vanadium resulted in one death. No symptoms of toxicity were observed at this dose level.

To be assured that the decrease in toxicity of vanadium in EDTA-pretreated mice was not simply a matter of local chelation in the peritoneal cavity, a group of 20 mice was injected with 1500 mg/kg $CaNa_2EDTA$, subcutaneously. Half of the group was injected with the LD_{70} dose of vanadium intraperitoneally 20 minutes later, and the other half was given the same dose of vanadium intraperitoneally 45 minutes later. Additional time was taken here to allow for the absorption of the EDTA salt from this site, which was slower than from the peritoneal cavity. Of the 20 mice prophylactically treated with the complexing agent, 4 mice of an expected 14 died, showing protection against vanadium administered by a different route, comparable to that when both agents are administered by the same route (Table II).

The optimal dose of EDTA to counteract vanadium toxicity was determined by injecting vanadium intraperitoneally at the LD_{70} level (10.9 mg V/kg). By maintaining the dose of vanadium constant and progressively reducing that of EDTA, in groups of 10 mice each, it was found that $CaNa_2EDTA$ at a dose of 62.5 mg/kg body weight protection first became significantly less than at higher dosage levels. At 30 mg/kg, $CaNa_2EDTA$ appeared to afford no protection (Table II, Col. 4-11). Additional groups of 10 mice each were used at certain critical points.

If 75 mg/kg is considered as the lowest dose of $CaNa_2EDTA$ which gives maximal protection against a vanadium dose 10.9 mg/kg, then it appears that approximately a 1:1 molar ratio of EDTA to vanadium is necessary for maximal protection prophylactically; seventy-five mg is one-fifth millimole of $CaNa_2EDTA$ (molecular weight, 374.3) and 10.9 mg is slightly more than one-fifth millimole of vanadium (atomic weight 51). Thus the optimal, prophylactic, protective dose of EDTA is one which is equal to or slightly greater than vanadium on a molar basis.

[†] Mice were obtained from Hamilton Laboratory Animals Inc., Cincinnati, O.

[‡] Values taken at experimentally determined points.

TABLE II. EDTA Protection against Vanadium Toxicity in Mice. Values in table are ratios of number of mice that died to number treated.

Vanadium, mg/kg	Mortality ratios									
	Controls (no EDTA)	$CaNa_2EDTA$, mg/kg intraper., 15 min. prior to vanadium inj.								
		1500	1000	500	375	250	125	75	62.5	30
18.3	19/20	10/20								
14.6	37/40	6/40								
10.9	28/40	1/10	0/10	1/10	2/10	2/20	0/10	2/20	7/20	6/10

Discussion. The mice that had been pretreated with EDTA showed the same symptoms after vanadium administration as those that had not been pretreated with EDTA. The symptoms usually consisted of depression and respiratory distress. In the non-pretreated mice, this depression usually progressed to a coma with clonic convulsions frequently occurring before death. The pretreated mice usually recovered from this depressed state in about 2 hours and appeared to have returned to normal within 24 hours. From this, it is concluded that in the EDTA pretreated mice, the vanadium is absorbed and exerts its initial toxicity. Some time later (about 2 hours) the metal chelate of EDTA is probably formed in sufficient amount to reduce the vanadium to a nonlethal level except in a few individuals.

The fact that *subcutaneously* administered EDTA protects against vanadium administered *intraperitoneally* shows that local chelation probably is not a significant factor in these experiments.

Summary. 1. Calcium disodium ethylenediamine tetra acetate has been shown to prevent fatalities in mice injected with LD_{70}, $LD_{92.5}$, and LD_{95} doses of vanadium as $NaVO_3 \cdot H_2O$, 80 to 90% of the mice injected at the LD_{70} and $LD_{92.5}$ dose levels of vanadium surviving. 2. At least one millimole of EDTA must be present for every millimole of vanadium in order to show maximal prophylactic protection. 3. The time required for EDTA to detoxify an LD_{70} dose of vanadium in mice appeared to be approximately two hours.

Acknowledgement is herewith given to Dr. H. E. Stokinger, Chief Toxicologist, Division of Occupational Health, U. S. Public Health Service, Cincinnati, for helpful suggestions and review of the manuscript.

1. Plumb, R. C., Martell, A. E., Bersworth, F. C., *J. Phys. and Colloid Chem.*, 1950, v54, 1208.

2. Long, F. A., Jones, S. S., Burke, M., *Brookhaven Conf. Rep.* BNL-c-8.

3. Martell, A. E., and Calvin, M., *Chemistry of Metal Chelate Complex Compounds* (Prentice-Hall) 1952

4. Rubin, M., *Pharmacology, Physiology, Biochemistry, and Toxicity of Versenes, Tech. Bull.*, Bersworth Chem. Co., 1952.

5. Bauer, R. O., *et al.*, *Fed. Proc.*, 1952, vII, 320.

6. Anonymous. *A.M.A. Arch. Ind. Hyg. and Occ. Med.*, 1953, v7, 137.

7. Priestly, J., *Phil. Trans.*, 1876, v166, 495.

8. Sjoberg, S., *Arch. Ind. Hyg.*, 1951, v3, 631.

9. Popovici, A., *et al.*, Proc. Soc. Exp. Biol. and Med., 1950, v74, 415.

Received May 26, 1953. P.S.E.B.M., 1953, v83.

Inhibition of Chicken Tumor I by Plasma from Chickens Infected with an Avian Malaria Parasite. (20356)

W. Trager and R. B. McGhee.

From the Laboratories of The Rockefeller Institute for Medical Research, New York City.

Certain similarities in the pattern of age resistance of chickens to tumor I of Rous(1,2) and to the malaria parasite *Plasmodium lophurae*(3) suggested that these two infections might influence each other in some way. No effect of the tumor on the course of the malarial infection was observed, but a series of experiments showed that the growth of the tumor was temporarily inhibited by the malarial infection(4). Preliminary experiments then indicated that plasma, taken from malarious chickens and injected intravenously into non-malarious chickens previously inoculated with a suspension of tumor tissue, might have an inhibitory effect on the tumor. This indication has been fully supported by the results of 5 successive experiments designed in the following way.

Materials and methods. For each experiment a group of day-old Rhode Island Red chickens was obtained and reared in a uniform manner. When the chickens were 2 weeks old, one of the authors (A) inoculated a suitable number of them with chicken tumor I (a strain which had been maintained in the laboratory of Dr. J. B. Murphy at the Rockefeller Institute and which was obtained through the courtesy of Dr. K. R. Porter). Each chicken received into each side of the breast muscle 0.2 ml of a suspension made by grinding in a glass grinder tumor tissue (from a young chicken inoculated 8 to 10 days previously) in nutrient broth in the proportion of 1 g of tissue to 2 ml of broth. The other author (B) then weighed the chickens and arranged them on the basis of weight into comparable groups. In each of the 5 experiments one such group was treated with normal plasma and a second with malarious plasma. In 4 of the experiments a third group was left without further treatment, and in one experiment a fourth group was treated with a second preparation of malarious plasma. The plasma samples were all prepared by worker A. For each treatment, 8 to 11 normal chickens of the same age as the tumor chickens, or 2 or 3 older chickens, depending on the experiment, were bled from the heart. For each 10 ml of blood, 1 ml of a solution containing, per 100 ml, 27 mg heparin and 0.85 g sodium chloride, was used as an anticoagulant. The individual blood samples were centrifuged and the plasmas were pooled. In a similar manner, pooled plasma was prepared from corresponding malarious chickens. These were in the 4th, 5th, or 7th day, depending on the experiment, of an infection with *P. lophurae*. Strain 12A of this parasite was used. It was passed in young chickens at 4- to 6-day intervals. Chickens to be used as donors of malarious plasma were inoculated with 0.2 ml, per 100 g body weight, of blood containing approximately 50 to 75 parasites per 100 red blood cells. The samples of pooled plasma, prepared by worker A, were turned over to worker B, who administered the treatments. Each treatment consisted of the slow intravenous injection of either 5 ml or 2.5 ml of the appropriate plasma to each chicken. On one or several days during the course of the experiment, worker A made an estimate of the relative size of the tumors. This estimate could not have been influenced in a subjective way, since worker A not only did not know which chicken received which treatment, but he also did not even know the arrangement of the chickens by groups. On the last day of the experiment (8 to 11 days after inoculation of the chickens with tumor tissue) blood films stained with Giemsa stain were prepared from the chickens which had been treated with malarious plasma. All of the chickens were then killed with chloroform, placed in paper bags, and left for several hours in a refrigerator. This treatment rendered the contents of the tumors less fluid. Worker A, still without knowledge of the grouping of the chickens, then cut into the breast muscle of each chicken, removed all of the loose mucoid tumor tissue which could be spooned and scraped

TABLE I. Effect of Intravenous Administration of Malarious Plasma on Mean Tumor Weight of Chickens Inoculated with Tumor I Tissue. 5 experiments.

Group	No. chickens	Treatment			Day[*] tumors weighed	Mean wt tumors, g	Diff. of mean		Ratio, mean tumor wt Group A/ mean tumor wt Group B
		Plasma	Dose, ml	On days[*]			g	p[‖]	
A	8	Normal[‡]	5	4–10	11	16	A − B = 7	.1	1.8
B	8	5-day m[†][‡]	5	4–10	11	9			
A	10	Normal[‡]	5	4–8	9	24	A − B = 10	.02	1.7
B	10	5-day m[‡]	5	”	9	14	C − B = 10	.02	
C	10	0	—	—	9	24	C − A = 0		
A	10	Normal[‡]	5	1–8	9	13	A − B = 5	.1	1.6
B	10	5-day m[‡]	5	”	9	8	C − B = 15	<.01	
C	10	0	—	—	9	23	C − A = 10	.02	
A	10	Normal[§]	2.5	4–7	8	16	A − B = 5	.1	1.5
B	10	4-day m[§]	”	”	8	11	C − B = 6	<.01	
C	10	0	—	—	8	17	C − A = 1		
A	10	Normal[§]	2.5	4–7	8	18	A − B = 7	.05	1.6
B	10	4-day m[§]	”	”	8	11	C − B = 6	.05	
C	10	0	—	—	8	17	A − D = 5	>.1	
D	10	7-day m[§]	2.5	4–7	8	13	A − C = 1		

[*] After day of inoculation with tumor tissue.
[†] m = malarious.
[‡] From chickens of same age as recipients.
[§] From 3-month-old chickens.
[‖] $p < 0.01$ for the differences between the means of Groups A and B in the entire series of 5 exp.

TABLE II. Body Weights and Tumor Weights of Individual Chickens of Exp. 5 (Table I) on the 8th Day after Inoculation with Tumor Tissue.

Treatment							
Group A Normal plasma		B 4-day malarious plasma		C No treatment		D 7-day malarious plasma	
Body wt, g	Tumor wt, g	Body wt, g	Tumor wt, g	Body wt, g	Tumor wt, g	Body wt, g	Tumor wt, g
215	22	185	12	234	21	222	14
264	26	241	2	232	5	212	19
273	9	246	16	246	21	250	16
225	20	243	7	224	17	275	10
287	29	272	13	281	6	280	10
252	5	229	14	249	14	261	14
224	17	209	9	236	17	255	19
153	12	222	9	175	14	209	16
248	6	221	8	232	31	250	14
295	29	314	19	280	21	290	2
Avg							
244	18	238	11	239	17	250	13

out, and weighed it. In this way there was obtained a quantitative measure of the amount of tumor development.

Results. The details of the treatments and the principal results of the 5 experiments are summarized in Table I. The consistency of the results, as expressed in the ratio of mean tumor weight of chickens treated with normal plasma to mean tumor weight of chickens treated with 4- or 5-day malarious plasma, is obvious. This consistency is reflected in the high degree of statistical significance of the differences between these means for the entire series of experiments ($p < 0.01$ as estimated from a calculation of $t(5)$). The low statistical significance of the difference in mean tumor weight between Groups A and B (Table I) in certain individual experiments is largely a result of the well-known inherent variability in the response of unselected chick-

TABLE III. Estimated Sizes of Tumors of Chickens of Exp. 3 (Table I) during Treatment.

		Sum of estimated sizes (± = 0.5, + = 1, 2+ = 2, etc.) on day after inoculation			
Group	Treatment	6	7	8	9
A	Normal plasma	17	19	24	22.5
B	Malarious "	7.5	8	15	18
C	0	20	23	29	32

ens to inoculation with tumor I. This variability is well illustrated in the results of Exp. 5, presented in detail in Table II. Table II also shows that there were no significant differences between any of the groups with respect to final body weight ($p > 0.9$). This was true in the other 4 experiments as well.

In only one experiment (Exp. 3) was there any difference between the mean tumor weight of the untreated group (C) and that of the group (A) treated with normal plasma (Table I). This also happened to be the only experiment in which the treatments were begun at one day rather than at 4 days after inoculation with tumor tissue. Interestingly enough, the effect of the normal plasma became apparent late in the relatively long course of treatment, whereas the effect of the malarious plasma was most noticeable early in the course of treatment (Table III).

In all the experiments, the estimates of tumor size made on the living chickens during the course of treatment showed that the tumors tended to become apparent later and to grow more slowly in the chickens treated with malarious plasma than in those treated with normal plasma or left untreated. For example, in Exp. 2, on the 7th day after inoculation with tumor tissue, 3+ to 4+ tumors were present in 2 of the chickens in Group B, 7 of the chickens in Group A, and 7 of the chickens in Group C. Again, in Exp. 5, on the morning of the 6th day and hence after only 2 treatments, the numbers of chickens in each group of 10 which still showed small growths (rated as ± or +) were: A (normal plasma) 3; B (4-day malarious plasma) 6; C (untreated) 3; D (7-day malarious plasma) 4. The remaining chickens in each group had tumors rated as 2+ or 3+.

With the dosages of malaria parasites which were used to infect the donors of malarious plasma, the malarial infection generally reached its peak on about the 6th day in young chickens and the 5th day in the older chickens. Hence all of the donors of plasma for the Group B chickens of each experiment were used shortly before the infection would have reached its peak. Since plasma taken on the 7th day of infection was used in only one experiment (Exp. 5, Group D) no definite conclusions concerning it can be drawn. However, the intermediate type of result obtained with it, or the possible complete lack of significant effect, was in keeping with a number of preliminary experiments which indicated that the most effective plasma could be obtained shortly before, rather than at or after, the peak of the infection.

Plasma obtained by the centrifugation of blood heavily infected with malaria parasites may be expected still to contain a few parasitized erythrocytes. These might be capable of initiating a malarial infection in the chickens which received large amounts of such plasma. Therefore the blood films prepared from these chickens on the last day of each experiment were each examined for malaria parasites for a period of 5 minutes. In this way, a few of the chickens were found to have very light infections. For example, in Exp. 3, in which the greatest amount of plasma was given, one parasite was found in the smear from one chicken and 2 parasites in the smear from another. It was known from the earlier work with chickens infected simultaneously with *P. lophurae* and tumor I that such light malarial infections had no effect on the tumor growth(4). *P. lophurae* was a fortunate experimental material from this point of view, for in chickens large doses of this parasite are required to produce a severe infection. Hence a few stray parasites introduced with the plasma in most cases produced no patent infection and could not have been responsible for the effects which were observed.

Discussion. It is evident that the plasma of chickens near the peak of an acute infection with *P. lophurae* exerts an inhibitory effect on tumor growth when it is injected into young chickens previously inoculated with chicken tumor I tissue. A simple hypothesis to explain this result would assume that there appears in the plasma of the malarious chickens an increased concentration of some nonspecific reaction product which has growth-inhibiting properties. An increase in lipoproteins has in fact been demonstrated in birds undergoing malarial infections(6). This may provide a clue as to the type of substance to be looked for.

It is of interest to note the recent observation(7) that the life of leukemic mice was prolonged when they were infected with *Plasmodium berghei,* a malarial parasite of certain rodents.

Summary. Two-week-old chickens were inoculated in the breast muscle with a suspension of chicken tumor I tissue. They were subsequently left untreated or given a series of intravenous injections of either normal chicken plasma or plasma from corresponding chickens which were near the peak of an acute infection with *Plasmodium lophurae.* On the day after the last treatment the tumors which had developed were weighed. In a series of 5 experiments, the mean tumor weight of the chickens left untreated or treated with normal plasma was 1.5 to 1.8 times greater than that of the chickens treated with malarious plasma.

1. Rous, P., and Murphy, J. B., *J. Exp. Med.,* 1914, v20, 419.
2. Duran-Reynals, F., *Yale J. Biol. and Med.,* 1941, v13, 61.
3. Trager, W., and McGhee, R. B., *J. Exp. Med.,* 1950, v91, 365.
4. Trager, W., *Fed. Proc.,* 1951, v10, 374.
5. Fisher, R. A., *Statistical Methods for Research Workers,* Oliver and Boyd Ltd., Edinburgh, 1944.
6. Trager, W., *J. Exp. Med.,* 1947, v85, 663.
7. Nadel, E. M., Personal communication.

Received May 27, 1953. P.S.E.B.M., 1953, v83.

Serum Complement Levels in Dogs Undergoing Kidney Homotransplantation.* (20357)

C. B. FAVOUR, JOSEPH E. MURRAY, C. T. WEMYSS, JR., A. COLODNY, AND BENJAMIN F. MILLER.

From the Departments of Medicine and Surgery, Peter Bent Brigham Hospital and Harvard Medical School, Boston.

Success with corneal(1), bone(2), and aortic(3) homotransplantation has further stimulated interest in the possibility of skin and renal transplantation in human medicine. Advances in surgical technics which have made possible autotransplantation of kidneys in experimental animals(4) as well as temporary homotransplantation in man(5) have focused new attention on the phenomenon of individual tissue specificity(6).

The mysterious failure of skin and kidney homotransplants days to weeks following an initial period of functional survival is suggestive of an immune response to an antigenic stimulus. Coincident with the development of experimental renal lesions induced by large doses of serum globulin a temporary depression of serum complement levels has been observed(7). Similar depressions of complement have also been noted in nephrotoxic nephritis(8) and human glomerulonephritis (9,10).† In the present study serial serum complement levels were determined in a group of dogs undergoing kidney transplantation. A comparison of these findings with patterns

---

* This research was aided by grants from the Bristol-Myers Pharmaceutical Co. and the National Institutes of Health of the U. S. Public Health Service.

† Suppression of circulating complement is not unique for renal disease. Studies of C. A. Janeway and co-workers (12) have shown this phenomenon to be a feature of "serum sickness" reactions produced by relatively pure antigens (albumin) which involve the arterioles. The most marked and sustained serum complement suppression observed in humans occurs in disseminated *lupus erythematosis* (11) with or without obvious renal disease.

seen in spontaneous and experimental renal disease is of interest for the understanding of the biology of organ homotransplantation.

Materials and methods. Homotransplantation. Healthy mongrel dogs weighing 10 to 14 kg were used as donors and recipients for kidney transplantation. Under nembutal anesthesia one kidney was removed from the prospective donor with preservation of adequate length of renal artery and of renal vein for anastomosis. In some earlier experiments, arterial perfusion of the donor kidney *in vivo* was done, using physiologic saline, with or without penicillin (5 cc with 5000 U/cc) and heparin (5 cc with 10 mg/cc). Since this procedure had no noticeable beneficial effect on the results, it was discontinued in later experiments. End-to-end suture anastomoses of the renal vessels to the iliac vessels were carried out in all cases reported (except Dog 47-52 where the neck site was used), and the ureter brought out to the skin. This location in the iliac fossa made possible gravity drainage of urine, a well anchored and protected graft, and helped prevent ureteral angulation. At the conclusion of the operation, the tip of the ureter bled freely, and clear urine appeared in a steady drip. *Complement determination.* Freshly drawn venous blood was allowed to clot at room temperature and the serum decanted within 90 minutes for storage at 8°C. The majority of determinations were carried out on the day blood was collected. Specimens examined at a later date were stored frozen at –20°C. No appreciable change in complement level was apparent under these conditions. Groups of sera containing specimens from normal controls were titered concurrently. A preliminary group of the complement titrations (Table I) was done by the 50% end-point method of Kabat and Mayer(13) modified by using a 5% instead of a 10% sheep cell suspension in the initial part of the procedure and by reducing the final volume of the test to 4 ml (2 ml sensitized cell suspension, 2 ml serum dilution) to facilitate hemoglobin determinations in the Klett-Summerson Colorimeter (5500 Å filter). Analysis of data on these 7 animals indicated that relatively high and variable readings resulted from the estimations in the upper range

TABLE I. Series I. Complement Levels in Dogs Undergoing Renal Homotransplantation.

Days	1		1				5					10					15				20			25				30
Dog 11	36			51	60		58		27	47	47			34	80*			51						76			74	
14	41				41		38	94	64	68	60			84				*		D								
35	51		51		60	86	65	57	61	55	60	57	74	73	·68	105	105	92	104	90		60						
47	59		67	80	94	90	82	100	88	120	140	140		110	110													
75	61			60				110	115	48		53		90		120		96			80					*		
92	66		48	29			86		130		160				145						130		106		105	115		118
98	55				70			90									62				50							
Avg	53	↑	55	58	64	88	66	90	81	67	93	83	74	78	101	113	84	80										
		Operation																										

* Graft removed. D = Animal died.

TABLE II. Series II. Complement Levels in Dogs Undergoing Renal Transplantation.

Days		3	2	1	1				5					10					15					20					25			Transplant	Avg
Dog 130		19	18	18	24	20	22	24		30	22	22	22	21	21	18	16		17	16	15	16	17	20	21	20	20	20	17	14	25	Auto-	
141				10	11	20	21	36	31	25	30	26	26	14	23	27	27	20	23	28	28	23	23	21								”	
131	AM		25	18		22	22	15	23	23	23	24	22	23	22		24	24	25	26	19		28	28	19*	22	36	30	20	27	22	Homo-	24
	PM		20		12	14	16	28	23	23	31	26	26	27	26	26	29	22	30	22		22										”	23
137	AM	12	14	14	14	20	22	26	31	24	19	19	23		28	31	25	26	26	22	18	19	22	12*	23							”	21
	PM			17	12	20	24	36		24	23	23	24		31	23	25															”	24
Avg		14	19	15	14	19	21	26	27	25	25	23	24	21	25	25	24	23	24	23	20	20	23	20	21								
Avg auto-				14																													
Avg homo-			22	18																													

* Graft removed.

of optical density. Taken as a group, however, readings occur in a normal distribution curve, following roughly the pattern obtained by the unmodified 50% end-point complement method used thereafter (Table II). The main experiment of this report was carried out on 4 dogs in which 2 homotransplants were compared with 2 autotransplants. The homotransplanted animals received a third kidney in the groin, and the autotransplanted animals had one of their own kidneys transplanted to the same location. Complement titers were determined in these 4 animals by the method of Kabat and Mayer without modification.

Results. All homotransplants stopped producing urine within 2 to 6 days. As urine flow ceased, the ureters retracted, blackened and finally disappeared into the wound. Coincidentally the transplant enlarged, forming a firm, palpable abdominal mass. Less commonly transplants disintegrated, leaving only remnants of necrotic tissue. The prompt establishment of urine flow and evident effective functional survival of the two autotransplants was in marked contrast to the course of events in the homotransplanted kidneys. Ten values on 8 normal control dogs,‡ and single preoperative values on the 7 animals operated upon, were employed to find the complement level of normal dogs (Table I). By the modified method the mean complement level was 53 units with a σ of 18 units. When the daily or the grouped postoperative values for the first 16 days are compared with the 17 normal values, no statistically significant change is noted.

In the second series of animals studied by the unmodified complement titration procedure, uniformly lower and more closely grouped values were obtained (Table II). The course of events for a typical experiment is given in Fig. 1. Normal dog complement for this method was found from 14 preoperative levels on 4 animals and single values on 2 other normal dogs.§ The mean for these 16 determinations was 23 with a σ of 4.5 units of complement. There is no significant differ-

---

‡ Values for normal dogs not operated upon are omitted in the tables.

§ Values for normal dogs not operated upon are omitted in the tables.

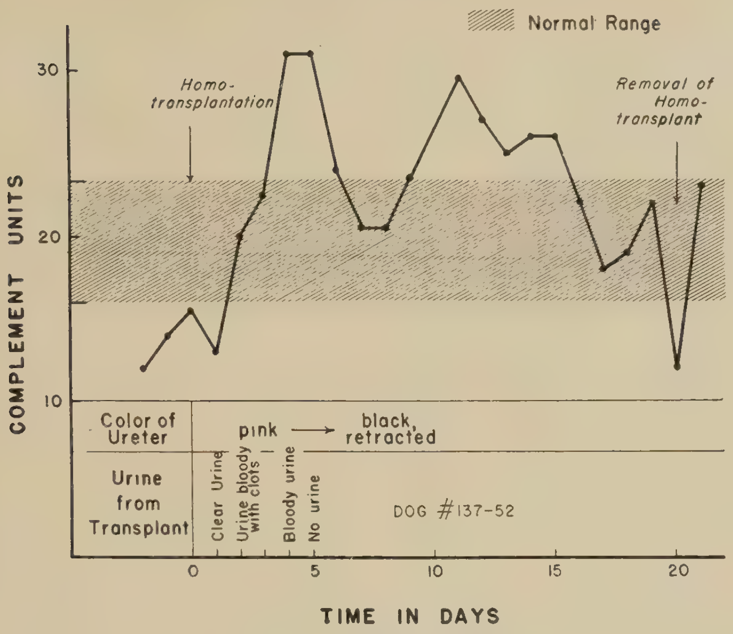


FIG. 1. Complement levels in a dog undergoing homotransplantation of a kidney into the iliac fossa.

ence between these 16 normal values and 30 postoperative values, days 1 through 16, for autotransplants ($t = 0.06$). On the other hand, a significant rise in complement followed homotransplantation (16 normal values compared to 56 values from days 1 through 16 give a t of 12.8 and P of >0.01). There is a significant difference between 16 normal values and values for many of the days after homotransplantation. The first day after operation there was a significant fall in complement ($t = 9.2$; $P = >0.01$). For the next 3 postoperative days complement lay within the normal range ($t = 1.8$; P 0.1. $t = 1.0$. $t = 0.8$). For days 5 through 16 complement was elevated significantly ($t < 4.0$; $P > 0.01$).

Discussion. In 9 dogs which were subjected to a kidney homotransplantation a uniform failure of the transplant was seen. In contrast to the 2 successful autotransplantations which functioned continuously following surgery, the urine flow of all homologous transplants ceased within 5 days. Various evidences of necrosis and infection promptly followed. Although there was a transient fall of complement on the first postoperative day in 2 of the 9 animals undergoing homotransplantation, the pattern of complement depression seen in human and experimental glomerulonephritis was not observed. This initial change could be due to many nonspecific causes. In these same animals a significant rise in complement in the late postoperative period was noted. During this later period the graft was undergoing dissolution. Similar complement rises associated with inflammatory processes have been seen by other observers(9). There was no change in complement postoperatively in 2 control dogs subject to autotransplantation. Neither was there a significant postoperative change in complement in 7 dogs undergoing renal homotransplantation who were studied with somewhat different method of complement titration. These patterns of complement levels after renal homotransplantation are quite unlike the "serum sickness" type of response in which a fall in complement ordinarily occurs a week or two after exposure to an antigen.

Summary. Homotransplantation of kidneys in normal dogs was followed by prompt renal shutdown and progressive failure of the graft. Frequent complement determinations were performed during the 3-week postoperative period. Unlike the situation in human glome-

rulonephritis and experimental nephritis a fall in serum complement 1 to 2 weeks later was not observed. Instead there was sometimes seen a significant rise in serum complement following homotransplantation.

The authors thank Doctor Frank Adler for valuable help.

Addendum

After this work was submitted for publication we learned that a similar study of serum complement levels in dogs undergoing kidney homotransplantation had been presented by M. Simonsen, Biological Incompatibility in Kidney Transplantation in Dogs, (E. Munksgaard. Copenhagen 1953).

---

1. Maumenee, A. E., and Kornblueth, W., *Tr. Am. Acad. Ophth.*, 1948, v52, 331.

2. Mawlem, R., *Brit. J. Surg.*, 1941, v29, 182.

3. Deterling, R. A., Coleman, C. C., and Parshley, M. S., *Surg.*, 1951, v29, 419; Gross, R. E., *Ann. Surg.*, 1951, v134, 753.

4. Williamson, C. S., *J. Urol.*, 1926, v16, 231; Dempster, W. J., *Ann. Royal Coll. Surg.*, 1950, v7, 295.

5. Hume, D. M., Merrill, J. P., and Miller, B. F., *J. Clin. Invest.*, 1952, v31, 640; *Am. J. Urol.*, in press.

6. Loeb, L., *The Biological Basis of Individuality,* 1947, C. C. Thomas, Springfield, Ill.

7. Schwab, L., Moll, F. C., Hall, T., Brean, H., Kirk, M., Hawn, C. V. Z., and Janeway, C. A., *J. Exp. Med.*, 1950, v91, 505.

8. Ogawa, S., and Sato, Y., *Tr. Soc. Path. Jap.*, 1938, v28, 212.

9. Fischel, E. E., and Gajdusek, D. C., *Am. J. Med.*, 1952, v12, 190.

10. Lange, K., Graig, F., Oberman, J., Slobody, L., Ogur, G., and LoCasto, F., *Arch. Int. Med.*, 1951, v88, 433.

11. Vaughan, J. H., Bayles, T. B., and Favour, C. B., *J. Lab. and Clin. Med.*, 1951, v37, 698.

12. Janeway, C. A., *Fate and Effects of Injected Protein Antigens. Nature and Significance of the Antibody Response,* 1953, Columbia University Press.

13. Kabat, E. A., and Mayer, M. M., *Experimental Immunochemistry,* 1948, 1st ed., C. C. Thomas, Springfield, Ill.

Received March 24, 1953. P.S.E.B.M., 1953, v83.

Phase Contrast Microscope Study of Oral Epithelium of Normal and Vitamin A-Deficient Rats.[*] (20358)

Louis J. Baume and Asger M. Frandsen. (Introduced by Karl F. Meyer.)

From the George Williams Hooper Foundation and the Division of Dental Medicine, College of Dentistry, University of California.

A recent investigation into the structure of the epithelial attachment to the tooth revealed that in fresh, undenatured oral epithelium prominent tonofibrils secure cohesion of the epithelial cells among themselves and with neighboring tissues such as the lamina propria and the surface of the tooth(1). These observations, made in man and monkey with the phase contrast microscope, suggested a corollary study of the behavior of the tonofibrils in oral epithelium of normal and vit. A deficient rats.

Experimental. Twenty male rats of the Long-Evans strain were fed a vit. A-deficient diet[†] beginning the week before weaning at 14 days of age. Their weight curves showed a plateau between the ages of 50 to 60 days. A maintenance dose of 3 μg of vit. A alcohol in oil was administered *per os* when they showed signs of extreme exhaustion. Four rats which survived the deficient diet for 86 days (100

---

[*] Aided by grants from the Research Board of the University of California and the California State Dental Assn.

[†] The vit. A deficient diet had the following percentage composition: vit.-free casein 24, sucrose 62, salts 4, cottonseed oil, vit. D, mixed tocopherol 10. The following vit. were added per 10 g diet: vit. D 10 USP units, tocopherol (alpha and delta) 3 mg, menadione 50 μg, thiamine 20 μg, pyridoxine 20 μg, riboflavin 50 μg, p-aminobenzoic acid 50 μg, niacin amide 100 μg, inositol 2 mg, choline citrate 10 mg, biotin 6 μg, pteroylglutamic acid 20 μg, pantothenic acid 350 μg, B_{12} 0.2 μg.

days old) were selected for the present study. A control group of 28 male rats were weaned and given a purified diet (30 μg vit. A alcohol to each 10 g of the deficient diet). Twenty of these rats were pair fed and used only for dietary evaluation. The other 8 rats were offered the control diet *ad libitum* and served in this study as normal controls when they were 100 days old. Fresh biopsies and briefly formalin-fixed specimens of the gingival papilla situated between the upper incisors were frozen (dry ice), sectioned and examined in a suspension of saline with the phase contrast microscope. Images of marked contrast can be obtained this way by illuminating the colorless, transparent objects according to the optical principles developed by Zernike(4). Photomicrographs were taken immediately.

Results. In the normal rat the dental papilla is bordered by a broad epithelial sheet (Fig. 1A). This epithelium consists of 10 to 15 cell layers and is occasionally enlarged by rete pegs at the free gingival margin. A wide stratum corneum is a physiologic characteristic.

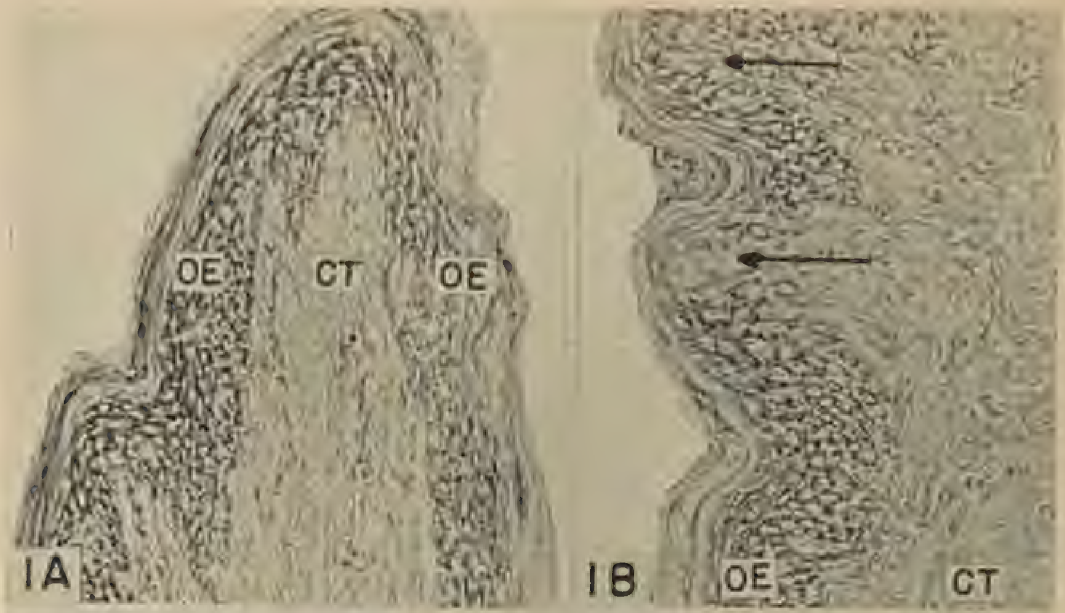
At high magnification a continuous network of tonofibrils is evident throughout the strata of living epithelial cells (Fig. 2A). A distinct structure conventionally called basement membrane adjoins the germinal layer of epithelium. This membrane is partly translucent and partly interwoven by reticular fibrils continuous with those of the lamina propria and the delicate tonofibrils of the basal cells of oral epithelium. These cells have voluminous nuclei containing prominent nucleoli and globules of chromatin (Fig. 3A). The nuclei gradually disappear toward the surface layers at the expense of an increasingly larger cytoplasm. Tonofibrils, seemingly looser oriented and finer in texture in the rat than in man and monkey, make up the bulk of the cytoplasm of these cells of the stratum spinosum (Fig. 4A). Their intracellular portions are somewhat compressed alongside the nucleus. Extracellular portions forming the intercellular bridges are only slightly larger than the intracellular parts and therefore give the entire network a rather homogenous appearance. As toward the outer surface of the epithelial sheet the cells become more dense and their nuclei no longer are seen, the tonofibrils disappear abruptly. Several layers of transparent, keratinized cells interconnected by few remaining intercellular bridges line the surface. The successive loss of cohesion results in the final shedding of the uppermost layer.

In oral epithelium of vit. A deficient rats some characteristic deviations from this normal pattern are observed. In general the entire epithelial sheet is thinner (Fig. 1B). A distinct border between epithelium and connective tissue is not discernible as the basement membrane is absent. At various places connective tissue appears to invade the epithelium reducing it to one single layer of living cells, the germinative cells; these contain exceedingly large nuclei (Fig. 2B). The life cycle of the epithelial cells is definitely shortened, evidenced by early karyolysis. However, in all viable epithelial cells tonofibrils are present and display the same morphologic features as in normal epithelium. Their connection with the reticulum of the connective tissue is obviously altered; without forming a network some tonofibrils fuse directly with delicate reticular fibrils of the connective tissue of the lamina propria (Fig. 3B).

Tonofibrillar continuity is lost already in the stratum spinosum (Fig. 4B). Before the nuclei have disappeared completely the tonofibrils decay into a granular mass. This granulated zone in the stratum spinosum is not encountered in normal epithelium and seems to be typical for vit. A deficiency. The succeeding keratinization is also disturbed, resulting in irregular and only slightly cohesive surface layers. It is noteworthy that this keratinous zone is approximately as thick as that of normal oral epithelium.

Discussion and conclusion. Undenatured oral epithelium of the normal rat possesses tonofibrillar structures similar to those in man and monkey(1). These tonofibrils, however, are finer in texture and more irregular in course, but are equally inscrutable to conventional histologic methods. Furthermore, the nuclei of the germinal zone of normal oral rat epithelium are much larger than in man; the keratinous surface zone is wider. Deprivation of vit. A engenders the following pathologic changes in oral epithelium: a) aplasia of the

PLATES I and II. Biopsies of gingival papilla between upper incisors of normal rats, 100 days of age (A) and vit. A deficient rats, 160 days of age (B) frozen sectioned in median sagittal plane at thickness 6-8 μ, suspended in saline, photographed without staining at various magnifications with phase contrast microscope.



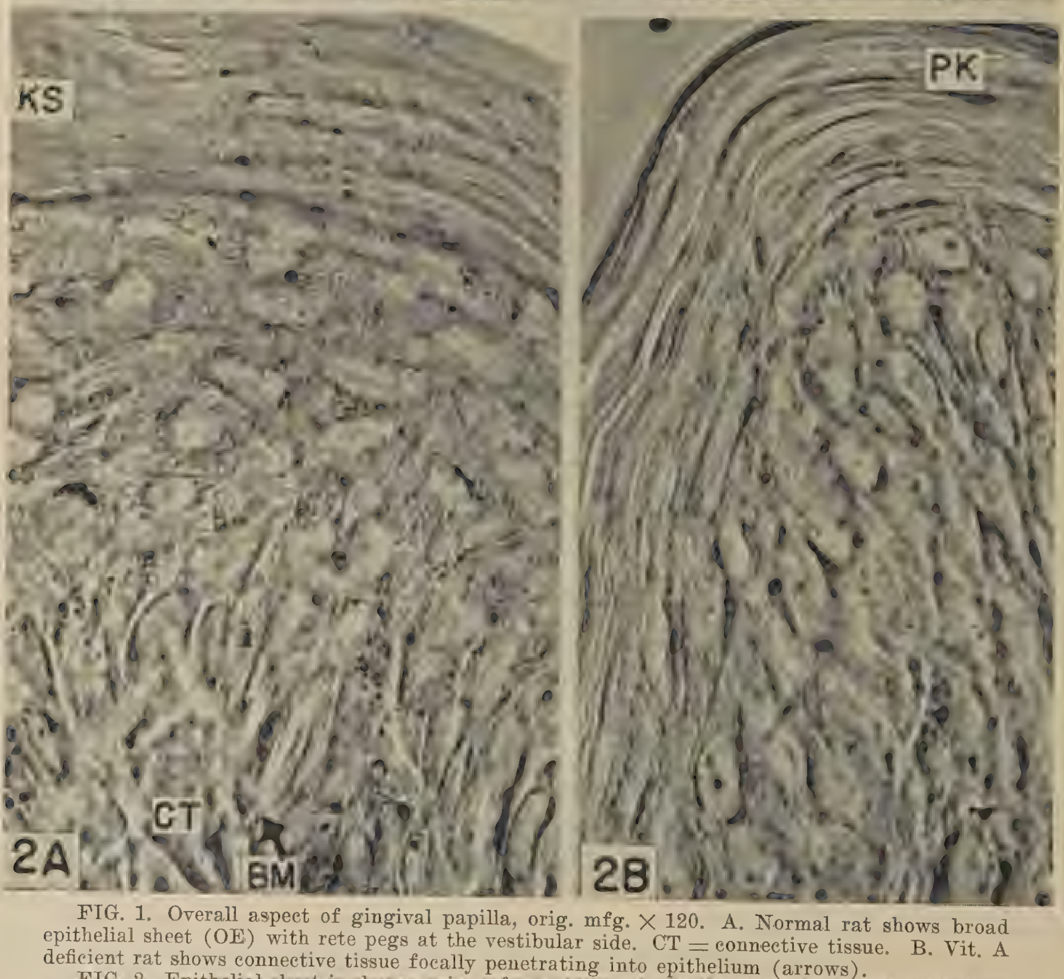
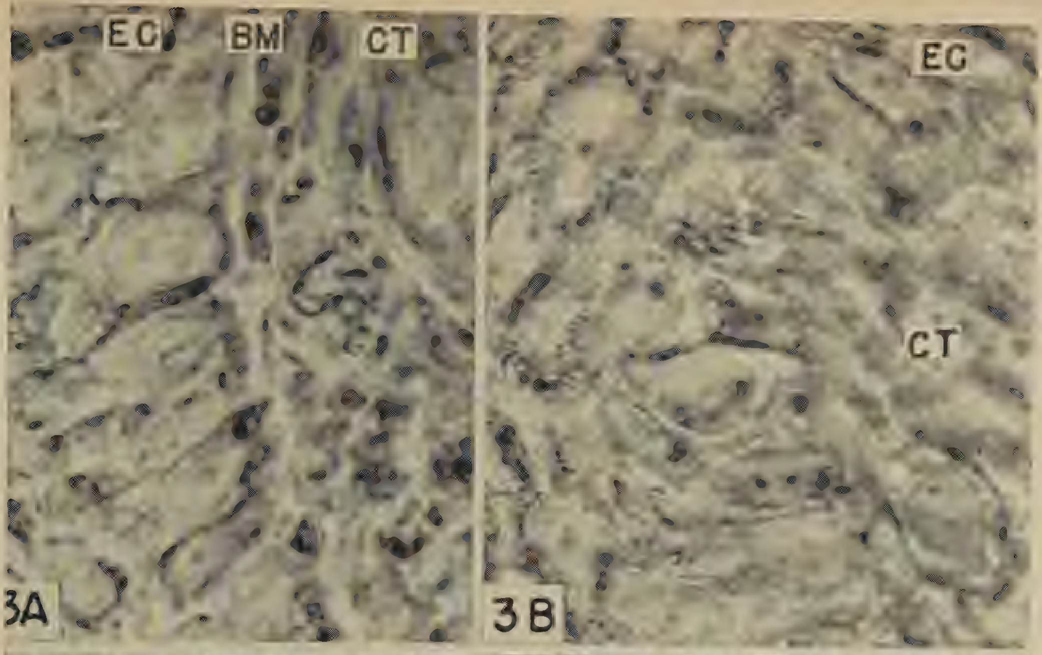


FIG. 1. Overall aspect of gingival papilla, orig. mfg. × 120. A. Normal rat shows broad epithelial sheet (OE) with rete pegs at the vestibular side. CT = connective tissue. B. Vit. A deficient rat shows connective tissue focally penetrating into epithelium (arrows).

FIG. 2. Epithelial sheet is shown, orig. mfg. × 1600. A. In the normal rat oral epithelium shows wide zone of keratinized cells at surface (KS), prominent tonofibrils in stratum spinosum and basement membrane (BM) at epithelio-connective tissue junction. B. In the vit. A deficient rat only few layers of living epithelial cells are present, the surface is parakeratotic (PK) and the epithelio-connective tissue junction is not discernible.



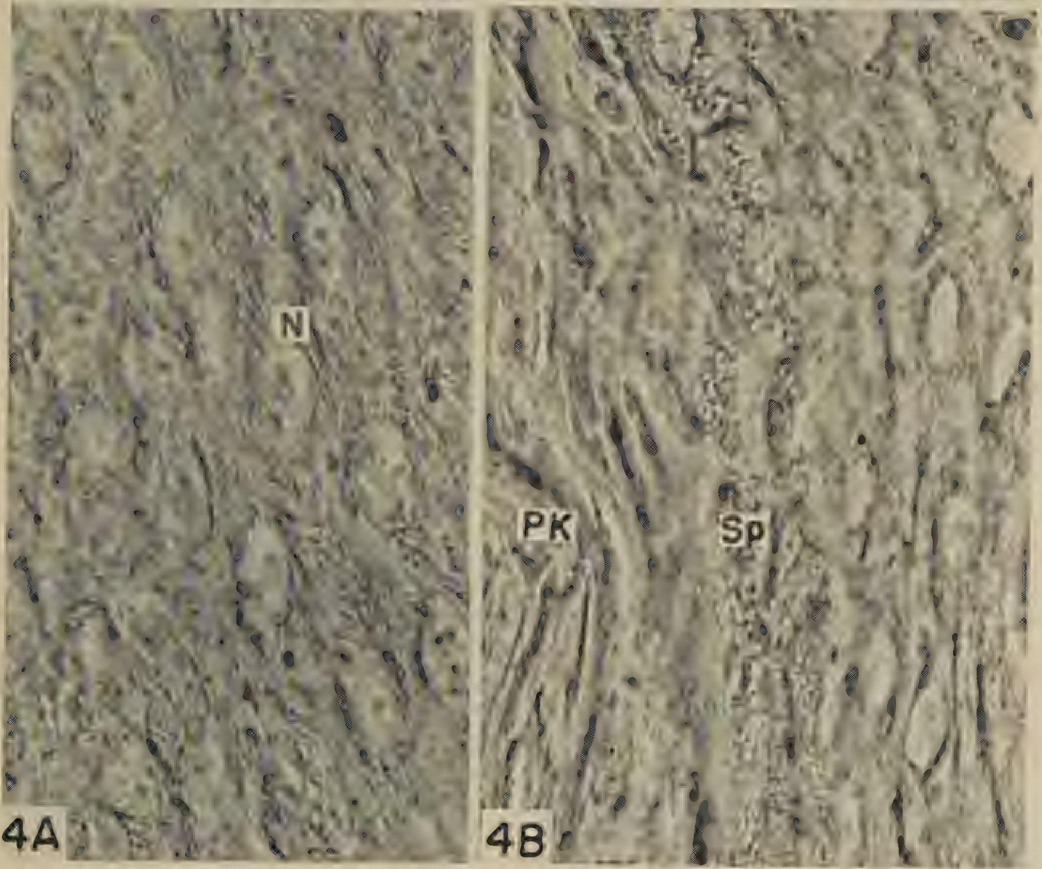


FIG. 3. Epithelio-connective tissue junction is illustrated, orig. mfg. × 2200. A. The normal rat shows translucent basement membrane interwoven with reticulum in continuity with tonofibrils of epithelial basal cells (EC). B. In the vit. A deficient rats few fine tonofibrils of basal cells fuse with delicate reticulum of connective tissue (CT) in absence of basement membrane.

FIG. 4. Stratum spinosum is shown, orig. mfg. × 1800. A. In the normal rat tonofibrils extend irregularly from one cell into another, compressed alongside the nucleus (N); the extracellular portions identical with the intercellular bridges are slightly enlarged forming desmosomes. B. In the vit. A deficient rat tonofibrils decay precociously effecting spongiosis (Sp) and parakeratinous surface layers (PK).

basement membrane at the epithelio-connective tissue junction, b) precocious decay of the tonofibrils in the stratum spinosum, and c) parakeratosis of the surface layers. a) Absence of a distinct border structure between epithelium and supporting connective tissue could indicate that one point of attack of vit. A deficiency possibly lies in the connective tissue. The basement membrane belongs to the ground substance of connective tissue and encloses a network of reticular fibers(2). The connection of this reticulum with the tonofibrils of the epithelium is altered in the mucosa of vit. A deficient rats. At various places connective tissue penetrates the epithelial sheet, limiting the latter to one layer of living cells. This points to a block in the trophic supply of the epithelium by the connective tissue in vit. A deficiency. The role of vit. A in the metabolism of connective tissue obviously warrants histochemical studies. b) Precocious, granular involution of the tonofibrils is related to the well-described metabolic dysfunction of epithelium in vit. A deficiency(3). Loss of tonofibrillar continuity within cells with a nucleus constitutes a specific disturbance that is not a stage of the normal cytomorphic changes of oral rat epithelium. Apparently it is identical with the histopathologic condition termed spongiosis. These observations on undenatured oral epithelium throw some new light on the nature of the prominent, but little known, protoplasmatic cell structures called tonofibrils. They corroborate the views previously expressed(1) that the presence of tonofibrils is bound to the vitality of the cell. Since vit. A, probably through intercession of connective tissue, controls differentiation and longevity of the epithelium, it directly or indirectly secures maintenance of tonofibrillar continuity. c) Keratinizing metaplasia, usually described as the main effect of vit. A deficiency, is a physiologic condition in oral rat epithelium. However, this keratinizing process is also disturbed in vit. A deficiency. Due to precocious decay of tonofibrils the surface layers are only slightly cohesive and very irregular in width.

Summary. Biostructures of oral epithelium such as tonofibrils and basement membrane were ascertained in normal rats with the help of phase contrast microscopy. Vit. A deficiency effected in oral rat epithelium 1) aplasia of the basement membrane, 2) precocious disintegration of the tonofibrils in the stratum spinosum, and 3) parakeratosis of the surface layer.

---

1. Baume, L. J., *J. Periodontol.*, 1953, v24, 63.
2. Gersh, J., and Catchpole, H. R., *Am. J. Anat.*, 1949, v85, 457.
3. Leitner, Z. A., Symposium on Vit. A, *Brit. J. Nutr.*, 1951, v5, 130.
4. Zernike, F., *Physica*, 1942, v9, 686.

Received April 21, 1953. P.S.E.B.M., 1953, v83.

Cooling of Embryonated Eggs to Produce an LD_{50} for *Coccidioides immitis.*

(20359)

JOSEPH R. GOODMAN, JOAN FOUNTAINE, AND JOHN VINCENT.
(Introduced by Charles M. Carpenter.)

From the Department of Investigative Medicine, Veterans Administration Hospital, Long Beach, Calif.

In studying a chronic disease process, an investigator is faced with the problem of choosing an objective criterion that is suitable for the evaluation of the progress of the disease or the effect of a therapeutic procedure. Procedures that utilize the evaluation of the LD_{50} are frequently used. However, agents that produce chronic diseases usually do not kill a large percentage of infected individuals in a relatively short period of time. Therefore, especially susceptible host species must be used or some other modifying procedure employed. Frequently physical agents such as X-ray(1) or chemical substances such as cortisone(2) have been used to decrease the resistance of the host and thus improve the probability of a successful invasion by the infective agent.

This laboratory has been studying *Coccidioides immitis.* This fungus when it disseminates in man produces clinical symptoms very similar to tuberculosis. It is highly infectious and can be established easily in experimental animals. The degree of involvement, however, is highly variable between individual animals. For example, identical inoculations of organisms into 10 rabbits of the same strain and age produced the following results: Two animals died of the infection in 6 weeks; 3 were chronically ill for 4 months and longer; 3 remained apparently healthy but at autopsy showed numerous widely disseminated lesions; and 2 showed no symptoms and no lesions at autopsy. Guinea pigs were more consistent in establishing the infection but varied considerably as to survival and clinical state of the infection. *Massive doses* of as many as one million arthrospores seldom produced death in eggs when inoculated into the allantoic fluid, although the mycelial stage of the organism was frequently observed on the chorion-allantoic membrane. When such massive doses do not cause death, an LD_{50} can be attained only by increasing the virulence of the invading organism, increasing the susceptibility of the host, or by using another host. Chicken embryos are particularly desirable hosts as they are generally initially sterile, are easily housed and maintained, create no diet problems, can be handled in statistically significant numbers, and are relatively inexpensive.

Methods. For these reasons it was decided to try to attenuate the chick embryos so that their resistance to the invading organism would be lowered and the LD_{50} thus attained. Although agents such as X-ray(1) and toxic chemicals(2) have been used, the simple procedure of cooling the eggs on their 9th day of incubation was employed. Eggs that have been incubated 8 days can be left in the disconnected incubator for 24 hours and still hatch without apparent ill effects on the 21st and 22nd day. The incubator in this laboratory was so located in the building that it cooled in 24 hours to 22°-24°C, as indicated by a maximum-minimum thermometer. The entire procedure consisted of incubating fertile eggs for 8 days at 38.5°C. After the viable eggs were allowed to cool to room temperature circa 26°-27°C, they were drilled, inoculated with *Coccidioides immitis* suspensions, and sealed. The *Coccidioides immitis* suspensions were harvested from mycelial growths that had developed arthrospores on Sabouraud dextrose agar. These slants were wetted with 5% glucose in saline solution. Great care was taken to minimize possible chance infection of personnel. After adequate material had floated free, the fluid was transferred to a sterile bottle containing glass beads. This was placed in a shaker to disperse the organisms further. This suspension was then tested for the number of viable spores by serially "diluting out" in liquid media and also by plating on Sabouraud dextrose agar. These counts were used to calculate the number of viable organisms in the suspensions used for inoculation of the eggs. When the egg incubator had cooled to about room temperature the eggs were returned and allowed to remain in the closed incubator until the following morning. The incubator was then turned on and the eggs candled. The eggs were candled each morning and the number of survivals noted. To minimize the danger of infecting personnel, all surviving infected eggs were killed just before hatching.

Results. Table I shows the number of cooled embryos that survived graded inoculations of *Coccidioides immitis.* LD_{50} dosage, calculated by the Reed-Muench formula(3), was 3500 spores, whereas 3 separate doses of 1 x 10^6 spores each on 3 different days in embryos that were not cooled produced no significant fatalities.

There were no significant fatalities of cooled embryos from the inoculation with autoclave-killed (½ hr-15 lb) suspensions of *Coccidioides immitis.*

Conclusion. 1. It is clear that the cooling of chick embryos on the 9th day has made it possible for the invading organisms to increase their effectiveness in overcoming the host. This technic has the advantage of a flexible and easily controlled attenuating device. The

TABLE I. Survival of 8-Day Eggs, Cooled 24 Hours, Inoculated with 0.1 cc Suspensions of *Coccidioides immitis*.

No. of 8-day eggs	Dosage	Days of survival in total age of embryo							
		9	10	11	12	13	14	15	16
46	1×10^6 spores	45	12	5	1	0	0	0	0
47	1×10^5	46	27	15	10	8	7	4	4
47	1×10^4	46	41	37	33	27	24	23	23
47	1×10^3	45	41	38	37	33	29	26	23
45	0.1 cc 5% glucose in saline	44	42	41	40	40	38	38	38

length of time and temperature of cooling can be chosen so that various degrees of susceptibility might possibly be attained if other invading hosts were to be used. 2. However, a word of caution is needed if chick embryos are to be used in a pseudo-quantitative manner to evaluate virulence or invasive capacity of an infective agent. Considerable variation on the hatchability or vigor of the embryos may occur from one region or one strain of chickens to another(4). Also nutritional factors of the mother hen, her age, and the phase of the egg laying season(5-8), all enter into the percentage of eggs that will hatch; hence, reflect the possibility of inherited variation of the susceptibility of the host.

1. Bennison, B. E., and Coatney, G. R., *J. Nat. Malaria Soc.*, 1949, v8, 280.

2. Kilbourne, F. D., and Horsfall, F. L., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 116.

3. Rivers, T. M., *Viral and Rickettsial Infections of Man*, Lippincott, Philadelphia, 1952.

4. Lacombre, Alta, and Summerland, B. C., *Empire J. Exp. Agric.*, 1946, v14, 25.

5. Couch, J. R., Cravens, W. W., Elvehjem, C. A., and Halpin, J. G., *J. Nutrition*, 1948, v35, 57.

6. Gillis, M. B., Heuser, G. F., and Norris, L. C., *J. Nutrition*, 1948, v35, 351.

7. Heywang, B. W., and Bird, H. R., *Poultry Sci.*, 1950, v29, 486.

8. Brown, W. O., *J. Sci. Food Agric.*, 1951, v2, 43.

Received April 23, 1953. P.S.E.B.M., 1953, v83.

Proteolytic Activity and Protein Catabolism of Rat Diaphragm in Hemorrhagic Shock.* (20360)

THEODORE B. SCHWARTZ.† (Introduced by Frank L. Engel.)

From the Department of Medicine, Duke University School of Medicine, Durham, N. C.

This work forms part of a study concerned with the biologic role of certain intracellular proteolytic enzymes. These peptidases, intensively and extensively studied by Bergmann and associates(1), exhibit a high degree of specificity so that it has been possible to measure accurately enzyme activity of highly inhomogenous preparations as a function of the rate at which, under standard conditions, a specific synthetic peptide substrate is hydrolyzed. Thus, glycylglycine‡ (GG) is hydrolyzed by a dipeptidase (GGase), present in many tissues, which has been shown by Smith(2,3) to be quite specific in its cleavage of this particular dipeptide.

That a state of shock serves as a potent stimulus to protein catabolic processes appears to be well documented. A review of these and other metabolic changes has been presented recently by Engel(4). The present investigation was undertaken to determine whether shock-induced acceleration of protein breakdown would be demonstrable in isolated rat diaphragm.

---

* This work was supported by grants from the National Institutes of Health of the United States Public Health Service, Duke Research Council and by the Medical Research and Development Board, Office of the Surgeon General, Department of the Army, under contract to Dr. Frank L. Engel.

† Damon Runyon Senior Clinical Research Fellow (1950-52).

‡ Provided through the courtesy of Dr. R. J. Floody, Hoffmann-LaRoche.

Materials and methods. After an 18-hour fast, adult male rats (Vanderbilt strain) were lightly anesthetized with Nembutal, 4 mg per 100 g body weight, and then bled from the cut tail. The bleeding was timed so that 3 to 3.5% of body weight was withdrawn in the first hour, a procedure which rather uniformly induces shock(5). The state of shock was maintained for the subsequent 2 to 6 hours. Terminal blood samples were obtained from the abdominal aorta followed by removal of the diaphragm. Plasma amino acid concentration was determined by a modification of the photometric ninhydrin procedure of Moore and Stein(6) with leucine as a standard. Throughout this investigation, control determinations on unbled animals were made concurrently with those on rats in hemorrhagic shock.

Experimental. GGase activity was measured in both extracted and surviving diaphragm. Aqueous extracts of diaphragm were prepared by grinding the isolated diaphragm in a mortar with a known volume (2.2 to 3 ml) of a medium(7) containing 103 mM of sodium chloride, 25 mM of sodium bicarbonate, 2.5 mM of calcium chloride, and 20 mM of sodium pyruvate. After centrifugation, the GGase activity of the clear supernatant solution was determined by a photometric ninhydrin technic(8). Dipeptidase activity of surviving diaphragm was measured by a procedure(9) in which the increment of free glycine is determined following incubation of washed isolated diaphragm with an excess of GG in the incubating medium. GGase activity, defined as μM of GG hydrolyzed per minute per g of diaphragm, was determined using a medium containing 42.8 μM/ml of GG and an incubation period of 15 minutes.

TABLE I. Effect of Hemorrhagic Shock on GGase Activity of Extracted and Surviving Rat Diaphragm.

	Extracted diaphragm		Surviving diaphragm	
	Control	Shock	Control	Shock
GGase* activity	2.25	2.00	.12	.12
S. E.	.184	.231	.004	.011
No. of animals	3	3	22	3

* GGase activity expressed as μM GG/ml of hydrolysis mixture, hydrolyzed/min./g of diaphragm.

As indicated in Table I, when compared with control values, there is no appreciable change in the GGase activity of either extracted or surviving diaphragm from rats in shock.

Results reported below support the conclusion that the metabolic effects of shock are secondary to tissue hypoxia. Since in the procedure for measuring GGase activity of surviving diaphragm(9), the incubated hemidiaphragm is repeatedly washed in media maintained in an *oxygen* atmosphere for one hour *prior* to incubation with substrate, it was conceivable that the incubated tissue "recovered" from shock during the preliminary exposure to oxygen, and, consequently, dipeptidase activity was found to be normal. Therefore, the experiment was repeated with only a 15-minute preliminary washing period. Again, the difference between GGase activity of diaphragms of 6 normal and 6 shocked animals was insignificant; the values obtained being 0.13 ± 0.021 and 0.09 ± 0.041, respectively.

In an effort to determine whether the protein catabolic effect of hemorrhagic shock was, in fact, demonstrable *in vitro,* net protein catabolism of normal and shock rats was studied. An amplification of a technic described by Kline(10) was used. Since, with this method, no exogenous substrate was added to the *in vitro* system, no specific proteolytic enzyme activity could be determined. Instead, net amino acid accumulation, which, in muscle, is taken to be a measure of net protein catabolism was measured. The freshly removed diaphragm was wiped free of blood on saline saturated gauze, and bisected. The control hemidiaphragm, after being weighed on a torsion balance, was immediately placed in 1% picric acid solution. The remaining hemidiaphragm was weighed and then placed in 2.7 ml of the medium described above, contained in a 20 ml beaker which had already been equilibrated with the 95% oxygen-5% carbon dioxide atmosphere in a Dubnoff metabolic incubator. The incubation was continued for a known interval of time at 37°C with shaking maintained at 90 cycles per minute. The hemidiaphragm was then removed, blotted on saline moistened gauze,

TABLE II. Effect of Hemorrhagic Shock on Net Protein Catabolism of Rat Diaphragm Incubated Aerobically and Anaerobically for 2 Hr.

Incubation atmosphere	Condition of animals	Diaphragm		Incubated medium	Net protein catabolism (NPC)	$\frac{D^*}{M}$	Terminal plasma amino N, mg %
		Control	Incubated				
		μM α-amino N per g diaphragm					
Oxygen	Normal	36.3 ± .6† (8)‡	17.6 ± .5 (10)	34.0 ± 1.2 (10)	16.4 ± .9 (8)	.53 ± .03 (8)	5.38 ± .17 (10)
	Shocked	35.0 (2)	17.5 (2)	31.6 (2)	14.0 (2)	.56 (2)	13.09 (2)
Nitrogen	Normal	37.4 ± .4 (3)	11.9 ± .8 (3)	48.2 ± 2.3 (3)	23.0 ±1.1 (3)	.25 ± .03 (3)	4.53 ± .23 (3)
	Shocked	35.4 ±1.7 (3)	10.4 ± .2 (3)	49.3 ± 2.2 (3)	24.3 ± .7 (3)	.21 ± .01 (3)	9.95 ±1.43 (3)
Oxygen	Normal	36.2 ± .5 (7)	14.8 ± .6 (7)	38.3 ± .6 (7)	16.9 ± .8 (7)	.38 ± .02 (7)	5.38 ± .29 (6)
Serial transfers to fresh media	Shocked	36.8 ± .9 (4)	11.4 ± .8 (4)	40.6 ± 1.0 (4)	15.2 ±2.3 (4)	.28 ± .02 (4)	13.9 ±1.29 (4)

Data above double line were obtained following uninterrupted incubation. The same procedure was followed to obtain the data placed below the double line except that incubated diaphragms were transferred to fresh media every 15 min. of the first hr of incubation.

$$^*\ \frac{D}{M} = \frac{\text{Incubated diaphragm } \alpha\text{-amino N}}{\text{Incubated medium } \alpha\text{-amino N}}.$$

† Mean ± stand. error. ‡ No. of animals.

and placed in 7 ml of 1% picric acid. Two ml of incubated medium were diluted to 10 ml with picric acid solution. The hemidiaphragms were finely ground and the resultant suspension sedimented by centrifugation. Non-protein α-amino nitrogen (AN) content was determined in the picric acid supernates of control hemidiaphragm, incubated hemidiaphragm and incubated medium by a photometric method(6). Leucine was selected for the preparation of standard curves and the values reported below represent, therefore, AN relative to the color yield of pure leucine. The sum of AN concentrations of incubated diaphragm and medium minus the AN concentration of the control hemidiaphragm (all expressed as μM AN per g diaphragm) is the AN concentration which accumulated during the time of incubation. This difference is referred to as net protein catabolism (NPC). Factors affecting NPC have subsequently been evaluated in detail and will be the subject of another report(11).

NPC was estimated first during a 2-hour period of incubation. Results are presented in Table II. The state of shock is reflected in the increased plasma AN concentration in the experimental group and this was a constant finding throughout the present study. Using the *two-hour* incubation period, it was apparent that NPC values of diaphragms from shocked animals were not appreciably different from those of unbled controls. This was true regardless of whether incubation took place in an oxygen or nitrogen atmosphere.

However, in both the normal and shocked state, the removal of oxygen from the incubation atmosphere resulted in striking changes. Anoxia was accompanied by a highly significant increase in NPC. In addition, the AN content of the incubated diaphragm fell and that of the medium rose so that the ratio of the two determinations fell from 0.53 in oxygen atmosphere to 0.25 under nitrogen.

Since it is held that all the metabolic concomitants of shock are the sequelae of tissue hypoxia(4), the increased "leakage" of amino acids from the anaerobically incubated diaphragm suggested that a similar change might occur aerobically in the shocked state. Accordingly, the comparison between NPC values of normal and shocked diaphragms main-

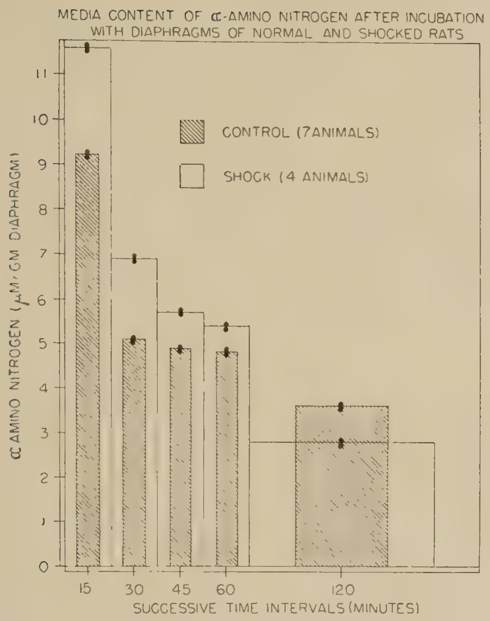


FIG. 1. See text for discussion. Black dots represent stand. errors.

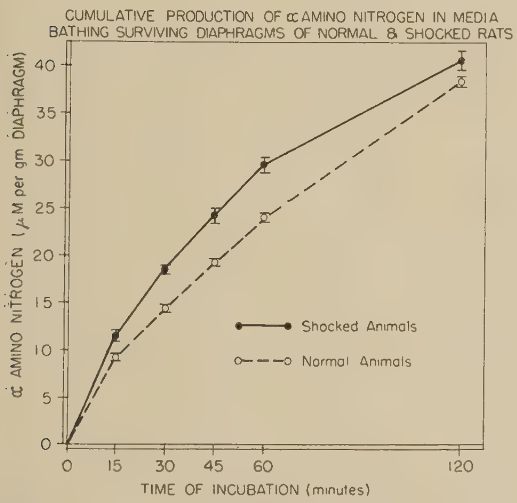


FIG. 2. See text for discussion. Vertical lines represent stand. errors.

tained in oxygen was repeated. In this instance, however, the incubated diaphragms were transferred to fresh media every 15 minutes for one hour followed by further uninterrupted incubation for the subsequent hour. Again, as recorded in Table II, there are only inconclusive differences between NPC values of normal and shocked tissue.

An examination of the serial media, however, proved to be fruitful as shown in Fig. 1. During the first 4 transfer periods, significantly more AN was released from the shocked rat diaphragms than from the normal controls. During the second hour of incubation, the reverse was observed so that the two effects cancelled each other over the total 2-hour period, as indicated in Fig. 2. Here, increments in AN content in the medium are plotted in a cumulative fashion. The increase in AN released from the shocked rat diaphragm remained consistently and significantly greater than that from the normal for the first hour. This increase fell off during the second hour so that, at the end of the 2-hour period, the differences became insignificant.

These findings led to the surmise that the determination of NPC after only *one hour* incubation might be profitable. The results, presented in Fig. 3, show clearly that under

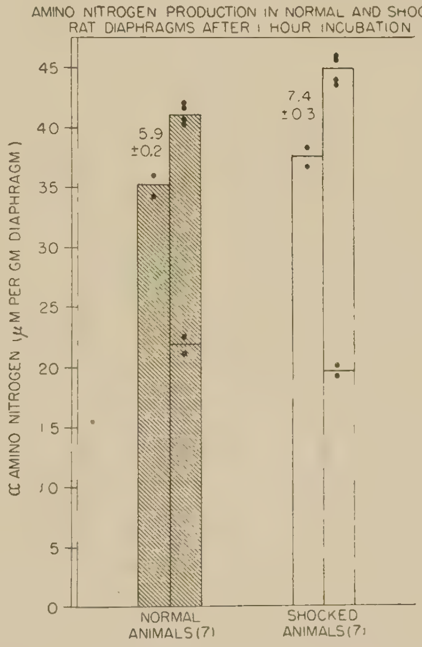


FIG. 3. Hatched column on far left represents AN content of control diaphragms from normal animals. Lower portion of second hatched column defines AN content of incubated diaphragm. Added to this is the AN content (per g diaphragm) of incubated medium. Difference between the heights of these 2 columns is 5.9 ± 0.02 μM/g diaphragm, the AN formed during incubation. Clear columns on the right are similarly charted. Black dots represent stand. errors.

these conditions, there is a relatively small but highly significant ($p < 0.01$) increase in NPC in diaphragms from shocked rats as compared with normal controls.

Discussion. There is convincing indirect evidence that increased protein breakdown in peripheral tissues is an integral part of the metabolic pattern found in hemorrhagic shock. Using the eviscerated rat, a preparation in which deamination of amino acids is negligible, Russell, Long, and Engel(12) showed that the rate of accumulation of AN in the blood was considerably greater in bled than in unbled animals. In dogs subjected to massive hemorrhage, Kline(13) found that the plasma AN arteriovenous difference became negative across the leg. The present demonstration that, after one hour incubation, the net protein breakdown from diaphragms of shocked rats is significantly increased above normal provides direct *in vitro* confirmation of the *in vivo* observations.

The report of Russell and Long(14) that the AN content of rat gastrocnemius was significantly increased following severe hemorrhage was not corroborated with the procedures employed in the present investigation, with rat diaphragm (Table II). The reason for this discrepancy has not been elucidated, but it should be recalled that the level of intracellular AN in muscle is dependent on membrane integrity(7) as well as intracellular protein metabolism. The present study provides clear-cut evidence of increased "leakage" of AN from diaphragms of shocked rats as well as increased protein breakdown in such tissues so that the "normal" AN content found may be the resultant of the 2 processes operating concurrently.

After the induction of hemorrhagic shock in the rat, its isolated diaphragm, in an oxygen atmosphere, behaves in a biphasic fashion with reference to release of AN; a period of relatively increased amino acid release, presumably the result of abnormal permeability, is followed by a period of decreased amino acid transfer. Further, during exposure to oxygen, the increased net protein breakdown in shocked rat diaphragm found after *one hour* incubation is no longer demonstrable if the time of incubation is doubled. Both of these observations may be interpreted as evidence of some degree of *in vitro* "recovery" from the shocked state. In view of the parallel metabolic effects of shock and anoxia, it does not seem unreasonable to attribute this "recovery" to the presence of high concentrations of oxygen in the metabolic incubator. Again, somewhat analogous observations have been made *in vivo*. Kline(13) found that the outpouring of AN from the leg of the dog, in hemorrhagic shock, could be reversed by replacing the blood previously removed.

Since, under properly selected circumstances, net protein catabolism of shocked rat diaphragm is enhanced, why are changes not discernible in specific peptidase activity? Various possibilities come to mind. First, it may be that shifts in the dynamic state which maintains protein mass are produced by changes in the rate of synthesis alone, without significant alteration in the rate of protein breakdown. Second, the highly specific dipeptidase chosen for study may not be representative of the many proteolytic enzymes which undoubtedly are involved in the catabolic process. Third, the negative nitrogen balance which occurs in shock could be provoked by relatively small increases in peptidase activity. Perhaps the presently available technics are simply too insensitive to reveal changes of this magnitude, although significant alterations have been produced(15) in GGase activity of surviving diaphragm following parenteral administration of an activator (cobalt) and inhibitor (cysteine) to rats.

Summary. The induction of hemorrhagic shock in the rat is accompanied by 1) no change in dipeptidase activity of extracted or surviving diaphragm; and 2) a significant increase in net protein catabolism of surviving diaphragm. Evidence is presented indicating that isolated diaphragms from shocked animals undergo some degree of "recovery" when exposed to an oxygen atmosphere.

The assistance of Mrs. Catherine C. Towbin is gratefully acknowledged.

---

1. Bergmann, M., *Adv. Enzymol.*, 1942, v2, 49.
2. Smith, E. L., *J. Biol. Chem.*, 1948, v173, 571.
3. ———, *J. Biol. Chem.*, 1948, v176, 21.

4. Engel, F. L., *Ann. N. Y. Acad. Sci.,* 1952, v55, 381.

5. Engel, F. L., Winton, M. G., and Long, C. N. H., *J. Exp. Med.,* 1943, v77, 397.

6. Moore, S., and Stein, W. H., *J. Biol. Chem.,* 1948, v176, 367.

7. Christensen, H. N., and Streicher, J. A., *Arch. Biochem.,* 1949, v23, 96.

8. Schwartz, T. B., and Engel, F. L., *J. Biol. Chem.,* 1950, v184, 197.

9. Schwartz, T. B., Engel, F. L., and Towbin, C. C., *J. Biol. Chem.,* 1952, v197, 381.

10. Kline, D. L., *Endocrinology,* 1950, v45, 596.

11. Schwartz, T. B., and Robertson, M. C., in manuscript.

12. Russell, J. A., Long, C. N. H., and Engel, F. L., *J. Exp. Med.,* 1944, v79, 1.

13. Kline, D. L., *Am. J. Physiol.,* 1946, v146, 654.

14. Russell, J. A., and Long, C. N. H., *Am. J. Physiol.,* 1946, v147, 175.

15. Schwartz, T. B., *J. Biol. Chem.,* in press.

Received May 4, 1953. P.S.E.B.M., 1953, v83.

Oxidation of L-Glutamic Acid by Rat Heart.* (20361)

Jorge Awapara.

From the University of Texas M. D. Anderson Hospital for Cancer Research, Houston, Texas.

Glutamic acid is known to be oxidized in tissues other than the liver and kidney and the enzymes responsible for its oxidation are known in a number of instances. Weil-Malherbe(1) for example observed that brain was able to oxidize glutamic acid. Dewan(2) isolated the enzyme and established its requirements. Independently from Dewan, von Euler and associates(3-5) made similar observations and reported an exhaustive study on glutamic dehydrogenase. In a large number of tissues, however, glutamic dehydrogenase exists in very low concentrations and could not account for the rapid utilization of glutamic acid. In heart muscle the glutamic dehydrogenase activity is very low or nonexistent(6), but glutamic acid is well utilized by homogenates of this organ, when fortified with ATP and coenzyme I. The results reported here clearly indicate that glutamic acid is oxidized only after transaminating with oxalacetic acid; the resulting α-ketoglutaric acid is then oxidized by way of the Krebs' cycle.

Methods. Male rats of the Sprague-Dawley strain and weighing approximately 250 g were used in all the experiments. The animals were sacrificed by decapitation, blood was drained off as much as possible, the heart removed and placed on a Petri dish resting over cracked ice. The muscle was freed of connective tissue and blood, then weighed and homogenized in a Potter homogenizer with ice cold isotonic KCl solution. The final concentration of the homogenate was 10%. In all experiments, 0.5 ml of the 1:10 homogenate was used in a final volume of 3 ml. The composition of the medium was identical to that given by Potter, Pardee, and Lyle(7) for the oxalacetic acid system, except that 500 γ of DPN were added in our experiments. The substrate concentration was 20 μM per flask unless stated otherwise. Oxygen uptake was measured by conventional Warburg methods at 38°C with air as the gas phase. *Ammonia* was determined in the deproteinized medium by distillation and nesslerization of the distillate. Aspartic acid and glutamic acid were determined by quanti-

TABLE I. Oxidations in Heart Homogenates.

Substrate (20/flask)	O_2 uptake 15m	30m	60m
No substrate	42	42	43
Glutamate	100	208	400
Succinate	60	120	219
α-Ketoglutarate	91	195	369
Oxalacetate	150	296	395
Citrate	42	46	54
Fumarate	80	160	273

---

* This work was supported by a grant from the National Cancer Institute of the National Institutes of Health, Public Health Service, and by American Cancer Society. Partly presented at meeting of Fed. Am. Soc. for Exp. Biol., Chicago, Apr. 1953.

TABLE II. Oxidation of L-Glutamic Acid by Heart Homogenates.

	Added (final conc.)		
Substrate	ATP	DPN	O_2 uptake in 1 hr
No substrate	.001 M		30 (26- 36) ^{4*}
Glutamate	”		80 (36- 90) ^{4}
No substrate		300	58 (50- 64) ^{4}
Glutamate		”	56 (48- 62) ^{12}
No substrate	”	”	45 (40- 46) ^{4}
Glutamate	”	”	385 (350-420) ^{8}

* No. of determinations.

TABLE III. Formation of Aspartic Acid from Glutamic Acid in Heart Homogenate. 5 experiments.

	Aspartic acid formed, μM	
O_2 uptake, μM	Theory	Observed
8.4	5.6	6.0
9.5	6.3	6.6
9.6	6.4	5.3
8.5	5.7	7.0
6.7	4.1	5.2

tative paper chromatography(8). The *L-glutamic* acid was obtained commercially and it was used as such.

Results. The ability of the heart homogenate to oxidize various intermediates of the Krebs' cycle was first established. The results are shown in Table I. With the notable exception of citrate all other components are utilized actively, including glutamic acid. Ammonia did not increase after the oxidation of glutamate, thus ruling out the possibility that glutamic dehydrogenase was involved. Furthermore, the addition of semicarbazide inhibited the oxidation of glutamate completely. In Table II are shown data obtained from several experiments. Glutamate was oxidized only when both ATP and DPN were added. The exclusion of either component resulted in a complete failure of the homogenate to oxidize glutamic acid. The concentration of ammonia was found to remain unchanged in all the experiments. Analyses of the reaction products by paper chromatography revealed that whenever glutamic acid was oxidized a substantial amount of aspartic acid was formed. The aspartic acid formed after the oxidation of glutamic acid was determined quantitatively and the results of several experiments are shown in Table III.

Few experiments were carried out to determine the effect of sodium monofluoroacetate on the oxidation of glutamate. Fluoroacetate is assumed to react with oxalacetic acid to form fluorocitrate. In the presence of fluoroacetate, the oxidation of glutamate was inhibited nearly completely. The inhibition, however, could be lifted by the addition of fumarate or malate.

Discussion. As far back as 1930, Needham(9) had observed that glutamate was oxidized by minced frog muscle to yield succinate without a concomitant reduction of amino groups in the system. Undoubtedly she was dealing with a transamination reaction. A similar type of oxidation has been described for aspartic acid by Nakada and Weinhouse(10). They used washed liver particles and observed that rapid utilization occurred. In the present study, a transamination is also responsible for the deamination of glutamic acid. There is probably sufficient oxalacetic acid present in the tissue at the start, which can and does transaminate with the added glutamate. The resulting α-ketoglutaric acid can be oxidized and yield more oxalacetic acid. This is probably the case since aspartic acid can be found in quantities which correspond closely to the theoretical amounts as calculated from the oxygen uptake. The inhibition by fluoroacetate could be interpreted as a removal of the oxalacetic acid by the inhibitor. In a recent paper, the author demonstrated that the injection of nonlethal doses of fluoroacetate resulted in a diminution of the concentration of free glutamic and aspartic acids in the heart(11). The present results and those obtained *in vivo,* however, are not comparable. The animal has other sources of keto-acids and there is always the added complication that blood can remove excess metabolites.

Summary. Glutamic acid was found to be oxidized by heart homogenates, by a mechanism involving transamination with oxalacetic acid and subsequent oxidation of the resulting α-ketoglutaric acid by way of the Krebs' cycle.

The author wishes to acknowledge the technical assistance of Mrs. Billie Seale.

1. Weil Malherbe, H., *Biochem. J.,* 1936, v30, 665.

2. Dewan, J. G., *Biochem. J.*, 1938, v32, 1378.

3. von Euler, H., Adler, C., Gunther, G., and Das, N. B., *Z. physiol. Chem.*, 1938, v61, 1938.

4. Adler, C., Gunther, G., and Everett, J. E., *Z. physiol. Chem.*, 1938, v14, 255.

5. von Euler, H., Adler, C., and Steenhoff-Eriksen, T., *Z. physiol. Chem.*, 1937, v248, 247.

6. Copenhaver, Jr., J. H., McShan, W. H., and Meyer, R. K., *J. Biol. Chem.*, 1950, v183, 73.

7. Potter, V. R., Pardee, A. B., and Lyle, G. G., *J. Biol. Chem.*, 1948, v176, 1075.

8. Landua, A. J., and Awapara, J., *Science*, 1949, v109, 385.

9. Needham, D. M., *Biochem. J.*, 1930, v24, 208.

10. Nakada, H. I., and Weinhouse, S., *J. Biol. Chem.*, 1950, v187, 663.

11. Awapara, J., *J. Biol. Chem.*, 1952, v197, 695.

Received May 8, 1953. P.S.E.B.M., 1953, v83.

Persistence of Amethopterin in Normal Mouse Tissues.[*] (20362)

JAMES R. FOUNTAIN,[†] DORRIS J. HUTCHISON,[‡] GEORGIA B. WARING, AND JOSEPH H. BURCHENAL.

From the Division of Experimental Chemotherapy, Sloan-Kettering Institute, the Chemotherapy Service, Memorial Center for Cancer and Allied Diseases, and the Sloan-Kettering Division of Cornell University Medical College, New York City.

In a previous report(1) it was shown that following the intravenous administration of amethopterin (4-amino-N^{10}-methyl-pteroylglutamic acid) to normal mice in a single dose of 0.5 mg/kg, the greater part of the compound was rapidly excreted or metabolized within approximately 4 hours. The remainder was found to be concentrated mainly in those tissues (liver and kidneys) which have been reported(2) as containing relatively high concentrations of folic acid and citrovorum factor.

As a consequence of this observation it was of interest to determine the persistence of the antimetabolite in various tissues after the administration of amethopterin, by various assay schemes, and furthermore to establish the identity of the anti-metabolite.

Materials and methods. The mice used in this experiment were of the inbred AkR stock, weighing approximately 20 g. All were young mice of approximately the same age. Groups of mice of different sexes were used. Previous to and throughout the experiment the mice were fed the standard Purina Laboratory Chow diet. For convenience of description the mice used in this study are divided into 6 groups based on the administration of amethopterin. *Group I* (31 mice) received amethopterin intraperitoneally at a dose of 3 mg/kg on alternate days for a total of 3 injections. *Group II* (18 mice) received 1 mg/kg on the above schedule. *Group III* (10 mice) received a single intraperitoneal injection of 3 mg/kg, and *Group IV* (12 mice) was given 1 mg/kg intravenously on alternate days for 3 injections. *Group V* (18 mice) was given amethopterin as for Group I. Seventy-two hours after the last injection 2 mice were sacrificed, and thereafter at daily intervals all the remaining mice were given citrovorum factor (CF)[§] intraperitoneally at doses ranging from 0.25 to 30.0 mg/kg. Mice were sacrificed daily 24 hours after last injection of CF. The procedures employed in obtaining and preparing tissues for assay were those previously reported(1) and the assay procedure was that of Burchenal *et al.*(3).

Group VI. For purposes of identification

---

[*] This investigation was supported by a research grant from the National Cancer Institute, of the National Institutes of Health, Public Health Service, by an institutional grant from the American Cancer Society, and by funds from the Damon Runyon Memorial Fund for Cancer Research, the Lasker Foundation and the Grant Foundation.

[†] Visiting Research Fellow of the British Empire Cancer Campaign and the American Cancer Society.

[‡] Research Fellow of the National Cancer Institute.

[§] Leucovorin (Lederle) supplied through the kindness of Dr. James Ruegsegger, Lederle Laboratory Division American Cyanamid Co., Pearl River, N. Y.

TABLE I. Concentration of Amethopterin (mγ/g) in the Liver, Kidneys, Spleen and Lungs of Mice over a 3 Week Period.

		Days after last inj.									
	Group	1	2	3	4	7	10	12	14	17	21
Liver (mγ/g)	I	338(3)	345(4)	410(3)	305(3)	384(2)	—	482(2)	427(2)	268(2)	426(3)
	II	294(4)	—	512(2)	—	408(2)	275(2)	432(1)	410(3)	—	375(2)
	III	332(2)	315(2)	—	309(1)	330(1)	368(1)	—	315(1)	—	284(2)
	IV	280(2)	—	412(2)	—	—	340(1)	350(1)	387(1)	261(1)	320(1)
Kidneys (mγ/g)	I	430(1)	453(2)	405(2)	430(1)	279(2)	—	327(2)	233(2)	218(2)	238(3)
	II	380(2)	320(2)	—	—	331(2)	260(1)	—	212(2)	—	101(2)
	III	—	—	—	290(1)	219(1)	231(1)	—	156(1)	—	126(2)
	IV	—	—	—	—	—	—	—	285(2)	380(1)	226(1)
Spleen (mγ/g)	I	0(1)	219(2)	140(1) 0(1)	0(1)	0(2)	—	0(2)	0(2)	0(2)	420(1) 0(2)
	II	0(2)	—	0(2)	—	0(2)	0(1)	—	0(3)	—	0(2)
	III	—	—	—	0(1)	0(1)	0(1)	—	0(1)	—	0(1)
	IV	—	—	—	—	—	—	—	0(2)	630(1)	0(1)
Lungs (mγ/g)	I	240(1)	80(2)	0(2)	0(1)	0(2)	—	0(2)	0(2)	0(2)	150(1) 0(2)
	II	150(1) 0(1)	—	0(2)	—	0(2)	0(1)	—	0(3)	—	0(2)
	III	—	—	—	0(1)	0(1)	0(1)	—	0(1)	—	0(1)
	IV	—	—	—	—	—	—	—	0(2)	475(1)	0(1)

Group I. Amethopterin 3 mg/kg × 3 i.p. alternate days. Group II. 1 mg/kg × 3 i.p. alternate days. Group III. 3 mg/kg i.p. single inj. Group IV. 1 mg/kg × 3 intrav. alternate days.

of the inhibitory substance in the liver, a series of mice was injected intraperitoneally with amethopterin at a dose of 3 mg/kg. These animals were sacrificed at ½ hour intervals up to 3 hours, then at 4, 5, 7, 17, 24, 48, 70 hours, 4, 8, and 14 days. Other mice not in this series were tested up to 8 months after injection. The liver was removed from each animal and treated as previously mentioned, after which the supernatant was concentrated *in vacuo* to approximately one ml. As controls for this experiment homogenates of 4 normal livers were prepared. Amethopterin (0.5 γ/g) was added to the first preparation, methopterin, N^{10}-methyl-PGA, (2 γ/g) to the second, aminopterin, 4-amino-PGA (1 γ/g) to the third, and to the fourth control was added a single preparation containing the 3 folic acid antagonists in the concentrations given above. These controls were boiled, clarified, and concentrated like the test samples. 10 μl of the liver concentrates were placed on Eaton-Dikeman No. 613 paper strips; these strips were developed for 6-68 hours in 3 different solvent systems.‖ After the strips were removed and air dried they were placed on large pyrex plates which contained Difco Folic Acid Assay agar plus PGA at 0.5 mγ/ml seeded with *S. faecalis* (ATCC 8043). The bioautographs of the inhibition zones were observed after an incubation of 16 hours.

Results. Table I shows the concentration of amethopterin in mγ/g of wet tissue in the organs of mice in Groups I-IV. Throughout the 21-day period mice in all groups showed the presence of amethopterin in the kidneys and liver. The levels remained remarkably constant, and the size of the dose bore no relationship to the amethopterin concentration. Similar results have been observed previously (1) following a single intravenous injection of 0.5 mg/kg and also in these experiments with a single dose of 20 mg/kg.

The serum was of interest in that amethopterin was detectable in all groups throughout the time of the experiments. The serum levels of Group I mice are shown in Fig. 1A, and similar levels were detected in Groups II-IV.

The serum level of amethopterin rose to significantly higher levels following the ad-

---

‖ 1% K_2HPO_4 aqueous(4)
5% NaCitrate and iso-amyl alcohol(4)
70% Iso-propanol, 10% NH_4OH, 20% distilled water. The paper strips were also buffered with 0.08 M Na_2HPO_4.

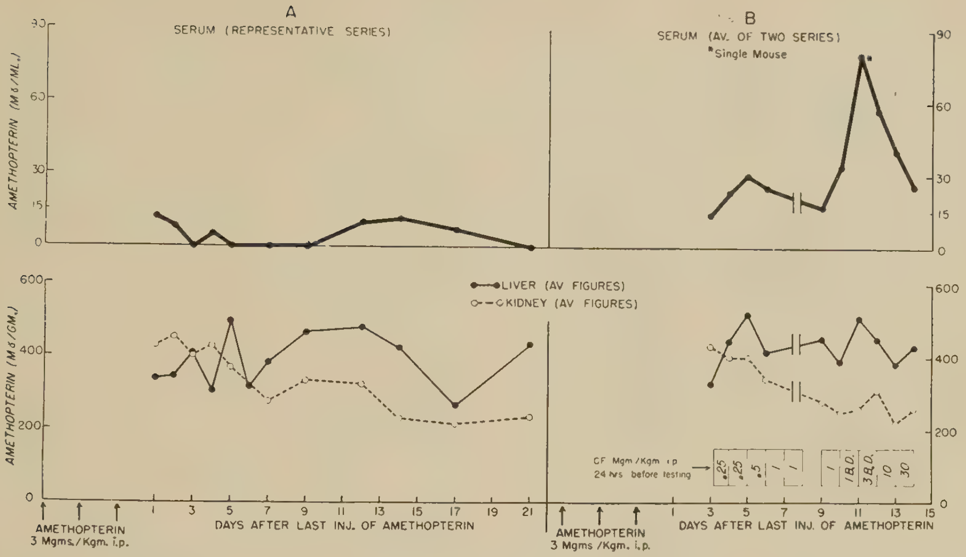


FIG. 1. Concentration of amethopterin in the serum, liver and kidneys of two groups of mice. (A) Amethopterin 3 mg/kg × 3 i.p., alternate days. (B) As for (A), followed by daily i.p. inj. of citrovorum factor.

ministration of CF (Fig. 1B). In order to be assured that the serum was relatively free of CF, it was not tested for amethopterin in the majority of instances until 24 hours following the injection of CF. The actual amount of CF administered did not appear to bear any relationship to the height of the amethopterin serum level.

It has been possible to identify the antimetabolite which persisted in the mouse liver and follow its course in a series of mice (Group VI). A bioautograph of a series of liver samples and of the clinical amethopterin (parenteral preparation) is presented in Fig. 2. Strip No. 1 shows the separation of aminopterin, methopterin and amethopterin, Strip No. 2 represents the particular sample of clinical amethopterin¶ that was injected into this series of mice. In the liver concentrates aminopterin was not detected after one hour and methopterin was present at 7 hours but not at 17 hours. It is possible that these 2 antifolics were excreted, but it is also possible that they cannot be detected because folic acid and CF have approximately the same Rf values as aminopterin and methopterin respectively. Amethopterin was detectable after 14 days, and in another series it could be identified by chromatography and bioautography after 8 months.

Identification of amethopterin in a liver taken 24 hours after injection is demonstrated in Fig. 3. Strip 1 shows the 3 spots representing aminopterin, methopterin, and amethopterin seen when these compounds were added to normal liver. Strip 2 shows the single spot found in liver of a mouse 24 hours after injection of amethopterin. When amethopterin was added to this liver preparation, only one spot was noted (Strip 3) but when methopterin (Strip 4) or aminopterin (Strip 5) was added a second spot appeared in addition to the original one representing amethopterin. Similar results have been obtained in the iso-amyl alcohol and K_2HPO_4 systems.

Discussion. The finding of 3 components in the amethopterin preparations complicates any metabolic study, but we have attempted to consider only the amethopterin content of tissues since neither of the other 2 components was detectable after 17 hours.

It has been noted that in normal volunteers (3) and in leukemic patients(5,6) variable

---

¶ Lederle Lot No. 7-8528.

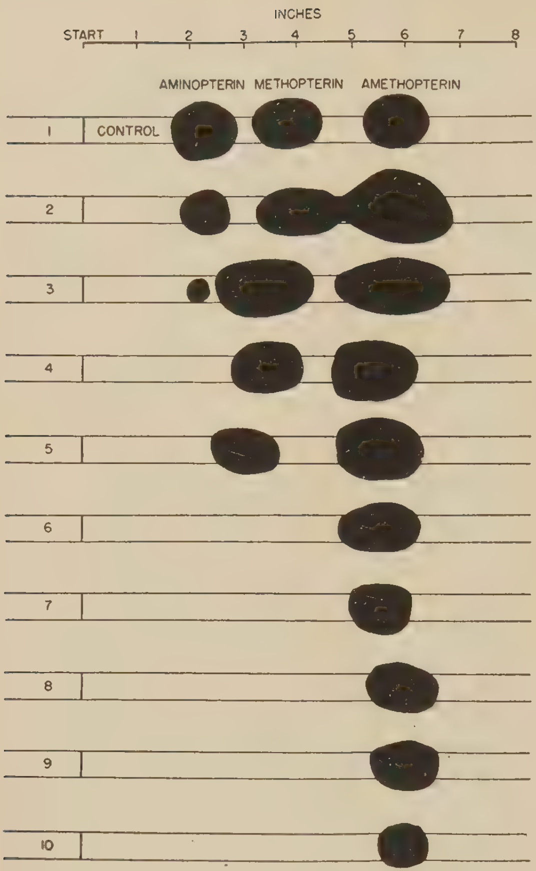


FIG. 2. Bioautographs of strips from alkaline iso-propanol system on *S. faecalis* (8043).

Strip 1. 10 μl of control liver concentrate which contained amethopterin, methopterin, and aminopterin (see text).
Strip 2. 100 mγ of amethopterin, Lederle Lot No. 7-8528.

10 μl of liver concentrates following administration of amethopterin (3 mg/kg).

Strip 3. After 1 hr
4. 2
5. 7
6. 48
7. 4 days
8. 8
9. 14
10. 8 mo

amounts of amethopterin or aminopterin cannot be accounted for by renal excretion alone.

It was evident from the experiments reported here that a relatively high concentration of amethopterin persists in tissues of the normal mouse for a period of 3 weeks and in one instance as long as 8 months in the liver. Moreover, it appeared that within the dosage range used in these experiments the mouse could rapidly eliminate within 24 hours all the amethopterin in excess of a threshold amount which is retained in the liver and kidneys. The effects of very large doses of amethopterin on tissue retention were not studied, as it was considered desirable to keep the dosage below the range which produces toxic manifestations(7) and within the chemotherapeutic range(8).

Although small quantities of amethopterin were found to be frequently present in the serum, there was little indication that any was being metabolized or excreted after the first 48 hours. Urine samples taken on several occasions at the time of sacrificing failed to show the presence of amethopterin.

In keeping with the hypothesis that antimetabolites function by excluding a structur-

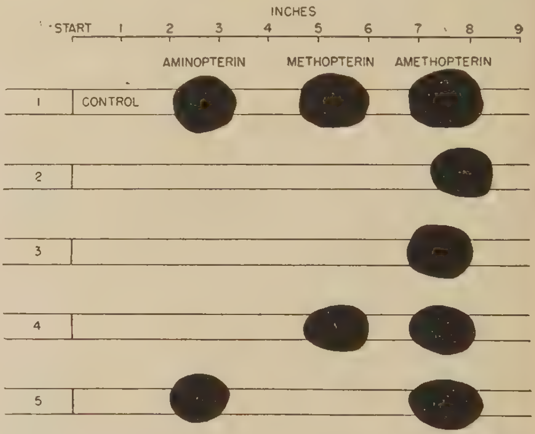


FIG. 3. Bioautographs of strips from alkaline iso-propanol system on *S. faecalis* (8043).

Strip 1. 10 μl n.l.h.* + 0.5 γ/g amethopterin (control).
15 μl n.l.h. + 2 γ/g methopterin (control).
10 μl n.l.h. + 1 γ/g aminopterin (control).
Strip 2. 10 μl l.c.* 24 hr after inj. of amethopterin (3 mg/kg).
Strip 3. 10 μl l.c. 24 hr after inj. of amethopterin (3 mg/kg) + 10 μl amethopterin control.
Strip 4. 10 μl l.c. 24 hr after inj. of amethopterin (3 mg/kg) + 15 μl methopterin control.
Strip 5. 10 μl l.c. 24 hr after inj. of amethopterin (3 mg/kg) + 10 μl aminopterin control.

---

* n.l.h. = normal liver homogenate; l.c. = liver concentrate.

ally related metabolite from combination with its enzyme(9) and thus preventing the formation of an enzyme substrate complex, it is probable that the amethopterin retained for such long periods of time is incorporated up to a relatively constant concentration in enzymes ordinarily associated with folic acid and CF, and any excess is rapidly excreted.

Summary. 1. Amethopterin given parenterally in various doses to normal mice resulted in the retention of a more or less constant amount of amethopterin in the liver for a period of at least 3 weeks. A decrease in the kidney concentration of amethopterin was noted. 2. CF at doses ranging from 0.5 to 30 mg/kg intraperitoneally failed to have any detectable effect 24 hours later on the concentration of amethopterin in the liver and kidneys, but caused a significant rise in the serum level of amethopterin. 3. The inhibitory substance present in the mouse liver 24 hours to 14 days after the administration of amethopterin has the same Rf value as amethopterin in 3 different solvent systems, and is therefore considered to be amethopterin.

We wish to acknowledge the technical assistance of Mrs. Elizabeth Robinson Hollands and the valuable assistance of Mr. V. M. Doctor in the chromatographic differentiation of amethopterin and methopterin.

---

1. Fountain, J. R., Waring, G. B., Hutchison, D. J., and Burchenal, J. H., PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 193.

2. Dietrich, L. S., Monson, W. J., Gwoh, H., and Elvehjem, C. A., *J. Biol. Chem.*, 1952, v194, 549.

3. Burchenal, J. H., Waring, G. B., Ellison, R. R., and Reilly, H. C., PROC. SOC. EXP. BIOL. AND MED, 1951, v78, 603.

4. Wieland, O. P., Hutchings, B. L., and Williams, J. H., *Arch. Biochem. and Biophys.*, 1952, v40, 205.

5. Swendseid, M. E., Swanson, A. L., Miller, S., and Bethell, F. H., *Blood*, 1952, v7, 302.

6. Ellison, R. R., Murphy, M. L., Hutchison, D. J., and Burchenal, J. H., Unpublished observations.

7. Ferguson, F. C., Jr., Thiersch, J. B., and Philips, F. S., *J. Pharm. and Exp. Ther.*, 1950, v98, 293.

8. Burchenal, J. H., Burchenal, J. R., Kushida, M. N., Jonhston, S. F., and Williams, B. S., *Cancer*, 1949, v2, 113.

9. Woolley, D. W., *A Study of Antimetabolites*, John Wiley and Sons, Inc., New York, 1952.

Received May 11, 1953. P.S.E.B.M., 1953, v83.

A Colorimetric Method for Hyaluronic Acid Estimation.* (20363)

W. A. PIERCE, JR., R. H. STEELE, AND A. G. C. WHITE.

From the Departments of Microbiology and Biochemistry, Tulane University School of Medicine, New Orleans, La.

Several methods are available for the quantitative measurement of hyaluronic acid. The material can be precipitated, purified, dried and weighed. The viscosity of its aqueous solutions can be measured, although this property varies, depending upon the amount of depolymerization that occurs during isolation and purification(1). The observation that hyaluronic acid forms an insoluble complex with protein at or near pH 4.0 led to the development of the turbidimetric test for hyaluronic acid(2). Although this technique has been widely employed and is currently used (with or without modification) for the measurement of hyaluronidase activity(3-6), the necessity for measuring the optical density of a suspension in which turbidity is increasing with time is a limitation. Under test conditions ordinarily employed turbidity formation proceeds rapidly at first and then at a decreasing rate for 10 to 20 minutes. The time elapsing between the addition of the reagents and the measurement of optical density must therefore be carefully controlled. Furthermore, when large amounts of polysaccharide are present flocculation may occur. The optical density then increases at first

---

* This work was supported in part by a grant from the U. S. Public Health Service.

but soon decreases as the floccules begin to settle to the bottom of the tube. Greif(6) has introduced a colorimetric method for hyaluronic acid which overcomes some of the difficulties. In his procedure, outdated blood bank plasma proteins are coupled to the indicator dye, bromsulfalein. When precipitation with polysaccharide occurs the dye is carried down in the protein-polysaccharide complex. After centrifugation the complex is dissolved in a standard volume of weak alkali. This releases the dye which then imparts to the alkaline solution a stable color that is proportional to polysaccharide concentration over a range of 0.1 to 0.2 mg.

The present report describes a simple colorimetric procedure for the determination of hyaluronic acid based on the co-precipitation of hyaluronic acid and hemoglobin. It is sensitive, accurate, and employs readily reproducible reagents.

Methods. Reagents. (a) 2M sodium acetate-glacial acetic acid buffer, pH 3.8. (b) Hemoglobin solution. This is prepared as follows: twenty-five ml of fresh sheep blood is centrifuged and the sedimented cells are washed three times with three volumes of 0.85% saline. The packed cells from the final centrifugation are suspended and laked in 50 ml of distilled water. After the addition of 2 ml of 25% aqueous sodium chloride, the erythrocyte stromata are removed by centrifugation at 8000 rpm for 30 minutes. The clear supernatant solution is then diluted with 100 ml of distilled water containing sufficient merthiolate to give a final concentration of 0.01%. Solutions thus prepared, if stored at 5°C, will give closely reproducible values in determination of hyaluronic acid for at least 3 weeks. *Substrate.* Streptococcal hyaluronic acid was prepared by the method of Harris and Harris(7).

Procedure. The test system consists of the following: 2 ml hemoglobin solution in distilled water; 0.5 ml sample; 0.5 ml distilled water; 2 ml 2M acetate, pH 3.8, listed in the order of addition of the solutions. The tubes (15 cm long x 11 mm O.D.) are allowed to stand in a refrigerated water bath at 5 to 10°C for one hour while precipitation is completed. The color of the mixtures changes gradually from red to brown as the hemoglobin is oxidized to methemoglobin. After centrifugation at 5-10°C for 15 minutes at 2700 rpm on a horizontal head, the supernates are poured off and the tubes drained 10 minutes in an inverted position. Excess fluid is removed from the rims with absorbent tissue. Five ml of distilled water and 2 drops of approximately 6 N NaOH are added and after the precipitates are redissolved with the aid of a glass stirring rod, approximately 0.02 g of crystalline sodium cyanide is added to each tube. The red color which develops has light absorption peaks at 420 and 545 μ. This suggests a similarity to or identity with ferriheme cyanide(8). Optical density is estimated on a Lumetron photoelectric colorimeter using a 550 μ filter having a 30 μ band width. Intensity of the color is maximal after 10 minutes and remains unchanged for at least one hour.

TABLE I. Hyaluronic Acid Recovery Tests.

Added	Found	Recovered	Max deviation
	mg		
.110	.135	.110	± .002
.110	.135	.110	
.110	.134	.109	
.110	.135	.110	
.440	.454	.429	± .011
.440	.463	.438	
.440	.454	.429	
.440	.463	.438	

Above amounts of purified hyaluronic acid were added to 0.5 ml aliquots of a culture supernate containing 0.05 mg hyaluronic acid/ml.

Preliminary trials to determine the optimal acidity for precipitation indicated that any pH between 3.5 and 4.2 would be equally suitable. The high molarity of the acetate did not interfere with precipitation and served to control effectively any changes in hydrogen ion concentration in the reaction system caused by the introduction of samples of different pH. Alkali and cyanide do not have to be measured with a high degree of accuracy. Considerable variation in the concentrations of these materials can occur without an apparent effect on the intensity of color developed with a given amount of hyaluronic acid. The results of recovery tests to determine the accuracy of the method are included in Table I.

For precise work a new standard curve

should be prepared at biweekly intervals and a standard solution of hyaluronic acid should be included in each determination as a reagent control.

Discussion. The procedure for determining hyaluronic acid described above has been found to offer a number of advantages not found in previously described methods. The reagents employed are easily prepared and readily reproducible. The hemoglobin solution can be standardized colorimetrically. The error is small, and a large number of tests can be carried out without the necessity for careful timing. Furthermore, when it is necessary to measure hyaluronic acid in broth culture supernates, the color of the medium does not interfere. A large series of heart infusion broth media, modified in various ways by the addition of sugars, buffers and growth factors, gave essentially identical blank readings whereas considerable variation would have been found if a turbimetric system had been employed.

A possible variable of the bromsulfalein method not present in the hemoglobin technic is the molar ratio of chromogenic substance (bromsulfalein) to protein. For good reproducibility this relationship must be constant. The molar ratio of heme to globin in native hemoglobin is a constant and thus fulfills this requirement. The accuracy and sensitivity of the method can readily be deduced from Fig. 1 which presents a standard curve with streptococcal capsular material. Color development follows Beer's Law closely over a range of 0.1 to 0.5 mg of polysaccharide. This gives a greater range of maximal accuracy than that found in either the turbidimetric or bromsulfalein procedures. Day to day reproducibility is improved by carrying out precipitation and centrifugation at a standard temperature.

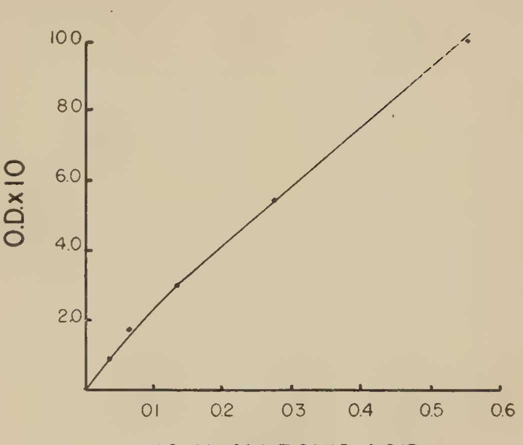


FIG. 1.

Summary. A new colorimetric method for the determination of hyaluronic acid is described, based upon precipitation of acid polysaccharide with hemoglobin at pH 3.8 and conversion of the prosthetic group of the precipitated protein to ferriheme cyanide. The color of this derivative is determined photoelectrically and the value for hyaluronic acid obtained by reference to a standard curve prepared with known material.

The authors wish to express their appreciation to Dr. William B. Wendel of the Department of Biochemistry, Tulane University School of Medicine for his helpful advice.

---

1. Meyer, K., *Physiol. Rev.*, 1947, v27, 335.
2. Seastone, C. V., *J. Exp. Med.*, 1939, v70, 361.
3. Kass, E. H., and Seastone, C. V., *J. Exp. Med*, 1944, v79, 319.
4. Dorfman, A., and Ott, M. L., *J. Biol. Chem.*, 1948, v172, 367.
5. Tolksdorf, S., McCready, M. H., McCullogh, D. R., and Schwenk, E., *J. Lab. and Clin. Med.*, 1949, v34, 74.
6. Greif, R. L., *J. Biol. Chem.*, 1952, v194, 619.
7. Harris, S., and Harris, T. N., *J. Immunol.*, 1949, v63, 233.
8. Hogness, T. R., Zscheile, F. P., Jr., Sidwell, A. E., Jr., and Barron, E. S. G., *J. Biol. Chem.*, 1937, v118, 1.

Received May 25, 1953. P.S.E.B.M., 1953, v83.

Influence of Cortisone on the Toxicity of Nitrogen Mustard in Rats. (20364)

Louise A. Hutchinson,* Gould A. Andrews, and Ralph M. Kniseley.

From the Medical Division, Oak Ridge Institute of Nuclear Studies, Oak Ridge, Tenn., under contract with the U. S. Atomic Energy Commission.

Cortisone and nitrogen mustard are sometimes combined in the therapy of lymphomas, leukemias, and other neoplastic conditions. There is relatively little information available on the combined effects of these 2 drugs. Nitrogen mustard and radiation are recognized to produce similar effects(1). A number of reports have been published on the action of cortisone on modifying the effects of total body irradiation. It has been reported that only 31% of mice receiving irradiation during cortisone administration survived, whereas there was 100% survival with either irradiation or cortisone alone(2), and a less pronounced but similar increase in mortality was noted in another laboratory in which cortisone was started immediately after irradiation(3). Thiersch(4) reports a delay in bone marrow regeneration in irradiated rats treated with cortisone. However, Mirand *et al.*(5) using mice found that cortisone afforded protection against radiation effects. Alpert, Zimmerman, and Scheer(6) making observations on combined therapy of malignant disease in humans observed that patients who failed to respond to either cortisone or nitrogen mustard alone, would not respond to the combination. However, their experience suggested that cortisone had some protective effect against the leucopenia resulting from mustard. In a discussion of the above work, Schoenback(7) observed that in experiments on mice he had not noted any reduction in the toxicity of mustard when used with ACTH or cortisone.

The following experiment was performed to observe the effects of cortisone on the LD_{50} (5-7 days) of HN_2 in rats.

Materials and methods. A pilot experiment showed that the LD_{50} dose of HN_2 administered subcutaneously was 1.2 mg/kg for male Wistar rats 5 months old and weighing 300-400 g. In the subsequent experiment a total of 60 rats were used. Two groups of 20 animals were started on cortisone 5 mg subcutaneously daily and a third group of 20 was started on control injections of .2 cc distilled H_2O subcutaneously. On the third day an LD_{50} dose of HN_2 was given to one group of 20 rats receiving cortisone and the group receiving control injections. The third group received only cortisone. All animals were observed for a period of 7 days following administration of the mustard, and during this time the daily cortisone and control injections were continued. A second experiment duplicating the conditions of the first was set up for the purpose of obtaining histological and bone marrow specimens. Animals were sacrificed on the second, fourth, fifth, seventh, and ninth days after injection of HN_2. Histologic sections of adrenal, spleen, and bone marrow were prepared. Smears of bone marrow were also made. The adrenals and spleen were weighed. In addition to these studies on sacrificed animals, similar information was obtained on some of those that died in both series.

Results. Table I gives the results of the mortality study. It shows no striking effect of the cortisone in either enhancing or protecting against HN_2 toxicity.

In the second experiment which was set up for the purpose of obtaining histologic data, the spontaneous death rate in the group of animals receiving nitrogen mustard alone was higher (13 animals) than in the group on HN_2 plus cortisone (8 animals). Because animals were being sacrificed at random in these groups, no effort was made to interpret this observation.

Bone marrow studies showed pronounced destruction on the second day and extensive depletion by the fourth day in Groups I and

---

* Present address: Department of Internal Medicine, Tulane University Medical School, New Orleans, La. Work done at Oak Ridge under Resident Physician contract.

TABLE I. Effect of Cortisone in Rats Given an LD_{50} Dose of HN_2.

Days after administration of HN_2	No. of rats dead Group I Mustard alone	 Group II HN_2 and cortisone	 Group III Cortisone only
0-3	0	0	0
3-4	1	2	0
4-5	4	6	0
5-6	5*	2	0
6-7	0	0	0
Total deaths	10	10	0
Survivors	10	10	20

* One traumatic death.

II which received HN_2 and HN_2 plus cortisone respectively. Persisting cells were largely plasma cells and reticulo endothelial elements. Megakaryocytes never disappeared completely. Mast cells remained plentiful. Starting on the fifth day regeneration was noted, and this progressed so that hyperplasia was present on the seventh and ninth days. In Group III (cortisone alone) the marrow picture showed no distinct changes during the experiment.

The spleens in all groups showed a definite shrinkage in size from the second through the ninth days. In the initial period this decrease in size appeared greater in Groups I and II than in Group III.

In Group III (cortisone alone) histologic studies showed preservation of the white pulp while in both other groups there was extensive atrophy of lymphatic tissue and a lack of evidence of activity of lymphopoietic centers of the follicles. There appeared to be some regeneration of the white pulp by the ninth day in Groups I and II, but this was minimal and did not compare with the striking regenerative change seen in the bone marrow.

The weights of adrenal glands indicated a definite shrinkage in Group III (cortisone alone) at intervals studied up through the ninth day. In the other 2 groups, changes in size were not striking or consistent except on the fifth day after the HN_2, when there appeared to be definite hypertrophy in Group I, with Group II being intermediate in size between Groups I and III.

Discussion. Under the conditions of the present experiment, the LD_{50} of nitrogen mustard was the same in rats receiving cortisone as in rats not receiving cortisone. The cortisone which was started 2 days before the HN_2 was given, failed to modify the severe bone marrow depression. The studies on the weights and histologic changes of adrenals and spleens were inconclusive because of the small number of animals used in the sacrifice experiments and because of difficulties in interpreting changes after spontaneous death. Both the cortisone and mustard appeared to cause splenic atrophy. In the adrenal the stress produced by the mustard resulted in enlargement which was maximum at the fifth day, and in the animals receiving both drugs this effect was partially neutralized by the cortisone which alone tended to produce atrophy.

This experiment represents an arbitrary set of conditions in the use of the 2 drugs together. Both have complex and widespread metabolic effects. It is likely that changes in the dosages and time sequence would produce results different from those which we obtained. It is entirely possible that some of the effects of cortisone are beneficial while others are harmful to the animal suffering from nitrogen mustard toxicity.

Summary. Cortisone failed to produce alteration in the LD_{50} of nitrogen mustard in rats under one set of experimental conditions.

1. *Advances in Internal Medicine,* 1950, v4, 24, Yearbook Publishers Inc., Chicago.

2. Wentworth, J. H., and Billows, J. A., *Radiology,* 1952, v59, 559.

3. Ellinger, F., *Pharmacological Studies on Irradiated Animals,* Part II, *Naval Med. Res. Inst.,* 1952, v10, 345.

4. Thiersch, J. B., Conroy, L., Stevens, A. R., and Finch, C., *J. Lab. and Clin. Med.,* .... v40, No. 2, 174.

5. Mirand, E. A., Reinhard, M. C., and Goltz, H. L., PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 397.

6. Alpert, L. K., Zimmerman, H., and Scherr, E., *Proc. 2nd Clin. ACTH Conf.,* 1951, v2, 235, Blakiston.

7. Schoenback, E. B., *Ibid.,* discussion of the work (6).

Received May 25, 1953. P.S.E.B.M., 1953, v83.

Role of Lipids in Formation of Yellow Pigment from p-Aminobenzoic and p-Aminosalicylic Acids. (20365)

R. L. Mayer, M. Grimm, D. Jaconia, and F. C. Kull.
From the Microbiological Research Laboratories, Ciba Pharmaceutical Products, Summit, N. J.

A number of micro-organisms are capable of producing yellow pigment (YP) when grown in the presence of p-aminobenzoic acid (PABA) and p-aminosalicylic acid (PAS) (1-7). The PABA pigment formed by mycobacteria is an oxidation product of PABA of still unknown chemical composition. The conditions under which it is elaborated, especially the influence of environmental changes on the amounts produced, and the sensitivity of the reaction to certain poisons, indicate the importance of oxidative enzymes in its formation. It is surprising therefore that among the many micro-organisms investigated the mycobacteria are by far the strongest YP producers in spite of their slow rate of metabolic activity. This may suggest that the mycobacteria, unlike the micro-organisms of other groups, possess specific mechanisms by which PABA may be oxidized into YP. An important difference existing between the mycobacteria and most other bacteria is the very high lipid content in the first group of organisms which may contain as much as 30% on a dry weight basis. Since the high concentration of lipids, of yet unknown physiological role, is a most distinctive biochemical characteristic of mycobacteria, we have investigated that part played by lipids derived from mycobacteria and other micro-organisms in the formation of YP from PABA and PAS. The ability of animal cells to form a YP from PABA was first described by Kohn and Liversedge(8). Subsequently Bernheim, Bernheim, and Wilbur(9) suggested that the factors responsible for this reaction are oxidized lipids or unsaturated fatty acids, most likely the peroxides or aldehydes and ketones formed during oxidation. When it became apparent that mycobacterial lipids were capable of forming YP from PABA, we extended our investigations to include lipids from various sources such as other bacteria, yeast, the higher plants and animals.

I. Formation of YP by various lipids. Materials. Lipids of Microbial Origin. a) Total lipids from *Mycobacterium smegmatis, Mycobacterium tuberculosis var hominis* (BCG strain) and *Torulopsis lipofera* were prepared by ether extraction of growth mats and centrifugates. b) Purified bacterial lipids were obtained from several sources. Dr. Hubert Bloch kindly supplied us with 6 lipid fractions derived from *Mycobacterium tuberculosis* which had been prepared during the isolation of the cord factor(10). Two esters of bacterial lipids were kindly made available to us by Dr. E. Lederer of Paris, namely methyl phtieonate, an α-β unsaturated acid, and the methyl-ester of coryno-dienic acid, isolated from *Corynebacterium diphtheriae. Lipids of Vegetable and Animal Origin.* Seventeen substances comprising non-drying, semi-drying and drying oils, hydrogenated oils as well as 5 lipids of animal origin (Table II) were tested. *Solvents.* All lipids were dissolved in a neutral organic solvent of low boiling point to yield concentrations of 0.5 to 10%; the solvent used was free of peroxides and similar impurities and did not possess oxidative or reductive properties. The most suitable solvent for most lipids was petroleum ether of C.P. grade. Other suitable solvents were alcohol, acetone and ether. Several of the purified bacterial lipids, insoluble in petroleum ether, were dissolved in ethyl acetate or chloroform. *Substrates.* PABA and PAS are soluble in alcohol or acetone, but insoluble in petroleum ether. When the latter vehicle (suitable for most lipids) was employed, finely pulverized PABA or PAS crystals were suspended in the lipid-solvent solution in a concentration of 10 to 20 mg per ml. For comparative studies it was preferable to employ more homogenous systems consisting of PABA esters (ethyl, butyl and isobutyl-p-amino-benzoates) which are soluble in petroleum ether. However, these esters reacted at a considerably slower rate than free PABA. In several assays PABA or PAS together with certain lipids were dissolved in alcohol or acetone. Although both solvents often contain

TABLE I. Yellow Pigment Formation by Microbial Lipids.

Lipids	Yellow pigment formation*
Total lipids:	
1. *Mycobacterium smegmatis*	+++
2. " *tuberculosis* (BCG strain)	+++
3. *Torulopsis lipofera*	+++
Purified lipids:	
1. Cord factor from *Myco. tuberculosis*	+
2. Non-toxic fractions isolated with cord factor	0
3. Methyl ester of phthienoic acid	+++
4. " " " corynodienic acid	+++

* These plus values represent relative amounts of YP produced by 10 mg PABA or PAS mixed with 5 mg lipid and exposed 4 days to air and filter paper. They correspond to colors produced by the following quantities of butter yellow dissolved in petrol ether and to colorimetric readings (filter 530 $m\mu$).

Dilution	mg/100 ml of solvent	Colorimeter light transm., %	Plus values	Color
1:200	500	18	+++++	Dark yellowish brown
1:400	250	31	++++	Light " "
1:1000	100	45	+++	" " "
1:2000	50	52	++	Amber
1:20000	5	83	+	Yellow
1:200000	.5	96	(+)	Straw yellow
1:2000000	.05	98	(±)	Faint "

oxidative constituents which accelerate the oxidation of PABA or PAS, the color differences between controls in which only PABA or PAS was present and systems which contained the lipids were nevertheless considerable.

Methods. Since only a few milligrams of the pure bacterial lipids and esters were available, a micro-method was devised which permitted the quantitative study of the development of the YP. This new method, similar to the filter paper technique previously described by Mayer, Grimm and Kull(11) for the analysis of water soluble oxidative enzymes, was based upon the following principle: Lipids and substrates (PABA, PABA esters and PAS) were thoroughly mixed and spread on sheets of No. 1 Whatman filter paper. The formation of YP on the filter paper could be quantitatively observed and the chromatic changes compared with color standards prepared by dissolving butter yellow in lard. In performing these tests sheets of the Whatman filter paper (22 x 18 inches) were ruled into 2 inch squares. With a 1 ml pipette, 0.05 or 0.1 ml amounts of the lipid-PABA solutions or suspensions described above were transferred to the center of a square and the solvent evaporated rapidly. The paper was then suspended in a dry atmosphere. The controls for each test consisted of solutions of the lipid material in the solvent, as well as suspensions of solutions of PABA of PAS in the solvent. *Fatty acids and oils* of which larger amounts were available, were tested in the following manner: 500 mg of fatty acid and 100 mg of PABA were dissolved in 3 ml of 1N NaOH, in a 25 ml Erlenmeyer flask, bringing the volume to 5 ml. The solution was then heated to the boiling point and the resulting yellow color compared with color standards. Two ml oil and 100 mg PABA were mixed in a test tube and heated in a boiling water bath until the color remained stable (15 to 20 minutes). Contrary to the filter paper method, this procedure permitted a more rapid estimation of the oxidative power of the lipids.

Results. a) *Controls.* All lipids used were colorless in the amount applied to the paper and no colors developed during several weeks of exposure to air. Areas containing PABA, and especially PAS alone, gradually became brownish but never formed YP. b) *Formation of YP by Bacterial Lipids.* Crude lipid

TABLE II. Yellow Pigment Formation with Various Fatty Acids, Vegetable and Animal Oils, and Fats.

Test substance (5 mg per test)	Pigment formation	
	5 days	10 days
Fatty acids		
Oleic	++	+++
Linoleic	++	+++
Stearic	0	0
Palmitic	0	0
Vegetable oils		
Non-drying		
Almond	(+)	++
Castor	0	(+)
Chaulmoogra	0	0–(+)
Olive	(+)	+++
Semi-drying		
Cottonseed	++	+++
Persic	++	+++
Rape	++	+++
Sesame	+++	+++
Drying		
Linseed	+++++	+++++
Poppyseed	+++	+++
Oiticica	+++++	+++++
Safflower	+++	++++
Walnut	+++	++++
Hydrogenated		
Palm	0	0
Cottonseed	0	0
Animal oils		
Sperm oil	0	(+)
Lard ”	(+)	+
Lard	+	++
Cod liver oil	++	++++
Ovolecithin	++++	+++++

extracts derived from *Mycobacterium tuberculosis* and *Torulopsis lipofera* were considerably active in transforming PABA and PAS into YP. The two unsaturated acid esters prepared by Lederer from Mycobacterium and *C. diphtheriae* also had strong activity, whereas Bloch's impure cord-factor was only slightly active. The various non-toxic lipid fractions obtained during the isolation of the cord-factor were inactive. The results are shown in Table I. c) *Formation of YP by vegetable lipids.* Most vegetable oils were capable of forming yellow pigment from PABA and PAS, but differed considerably in their pigment forming activity. In general, the activity was directly proportional to the drying properties of the oils. In the group of non-drying oils, only castor and chaulmoogra oils were virtually inactive; almond, olive and palm oils demonstrated weak activities, while peanut oil proved to be a good yellow pigment former. The 4 semi-drying oils were strongly active. The highest activities were found among the drying oils since linseed, oiticica and tung oils formed YP almost immediately upon contact with PABA. Hydrogenated palm and cottonseed oils had completely lost their activities. The results obtained with 5 mg amounts of these vegetable oils mixed with 10 mg PABA or PAS are noted in Table II. d) *Formation of YP by animal Lipids and Fatty Acids.* Animal oils and fats had various degrees of activity. Sperm oil was practically devoid of activity, while lard and lard oil had good activity, and cod liver oil extremely high activity similar to that of drying vegetable oil, oiticica and tung oils. Ovolecithin was found to be very active, producing YP in less than 24 hours. Among the fatty acids, oleic and linoleic acids were powerful oxidizers of PABA and PAS, whereas saturated fatty acids such as stearic and palmitic acids were completely inactive. The results are inscribed in Table II. It has been shown in these experiments that many bacterial, vegetable and animal lipids and certain unsaturated fatty acids are capable of forming YP from PABA and PAS. With the vegetable oils, this activity paralleled the drying properties of the oils. It was therefore desirable to investigate the mechanism involved in this reaction.

II. Assays to determine the active principle in lipids forming YP from PABA and PAS. Wilbur, Bernheim and Shapiro(12) have found that the color reaction of thiobarbituric acid in the presence of oxidized unsaturated fatty acids which parallels the formation of YP from PABA may be produced by peroxides or ketones and aldehydes. However, certain chemical characteristics pointed to aldehydes or ketones rather than to peroxides as the principal agents involved. The transformation of PABA and PAS into YP may be brought about under the following conditions: 1) the presence of double bonds within the lipid or fatty acid molecule, which may act as hydrogen acceptors; 2) the presence and autoxidative formation of peroxides at the ethnoid linkages of the unsaturated fatty acids; 3) the presence of other oxidation products such as aldehydes and ketones, formed either from peroxides or in addition to peroxides. The *oxidative potentials* of these 3 systems are

TABLE III. Influence of "Blowing" (Oxidation) and Heating of Various Lipids and Oleic Acid on YP Formation.

Test substance	Treatment	Ability to form YP after: 48 hr	96 hr	Peroxide No.
a. Microbial lipids				
Myco. smegmatis	0	+	+++	—
	ox*	+++	++++	—
" *tuberculosis*	0	+	++	—
	ox	++	+++	—
Torula lipofera	0	+	++	—
	ox	+++	+++	—
b. Non-drying oils				
Almond	0	0	(+)	49
	ox	(+)	+	303
Castor	0	0	0	10
	ox	0	0	18
Chaulmoogra	0	0	0	22
	ox	(+)?	(+)?	236
Olive	0	+	+	25
	h	++	++	12
	ox	++	++	779
	ox + h	++	++	6
c. Semi-drying oils				
Persic	0	+	++	20
	ox	++	+++	211
Cottonseed	0	+	++	116
	h	++	++	20
	ox	++++	++++	646
	ox + h	+++++	+++++	22
Rape	0	+	+++	20
	ox	++++	++++	120
Sesame	0	+	+++	38
	h	+	+++	17
	ox	+++++	+++++	863
	ox + h	++++	+++++	23
d. Drying oils				
Linseed	0	+	+++++	7
	ox	+++	+++++	101
Oiticica	0	++	+++++	12
	ox	+++++	+++++	160
Poppyseed	0	+	++++	35
	ox	+	++++	694
	ox + h	++++	+++++	0
Walnut	0	+	++++	21
	ox	+++	++++	247
Cod liver	0	++	++++	23
	ox	++++	+++++	74
e. Fatty acid				
Oleic	0	+	++++	40
	ox	+++++	+++++	375

* 0 = untreated; ox = oxidized; h = heated.

different and it is probable that they attack PABA or PAS with different degrees of facility. In order to recognize which factor may actually effect the formation of YP from PABA and PAS, we enhanced the various oxidative principles in a number of various oils. This was achieved by 1) heating at 165°C, 2) *"blowing"*, and 3) *"blowing"* and subsequent heating at 165°C without aeration. Heating at 165°C slightly increased the peroxide content. *"Blowing"*, the technical procedure employed to increase the viscosity of drying oils, consists of heating the oils at 125°C and passing air through the oils during the heating process for various periods of time. In our experiments, the oils were "blown" with a vigorous stream of air for 24 hours over a steam bath. This procedure considerably increased the viscosity of semi-drying and drying oils, in some instances to an almost resinous consistency. The polymerization of these oils is due to an intense oxidation during the "blowing" process, with an appreciable resultant increase in peroxide content. Subsequent heating of the "blown" oils at 165°C for one and one-half hours reduced the amount of peroxides to the original level with formation of highly reactive aldehydes and ketones. Oils treated by the above procedures were tested for YP activity. Bacterial lipids were oxidized by irradiation with ultraviolet light as indicated by Wilbur, Bernheim and Shapiro(12). As in the preceding experiments, 5 mg of lipid material was mixed with 10 mg PABA or PAS, spread on filter paper and exposed to air.

As seen from Table III, the oxidation of lipids from *Mycobacterium smegmatis, Mycobacterium tuberculosis var. hominis* (H37-Rv) and *Torula lipofera* increases their ability to form YP. Oxidation of non-bacterial lipids has variable effects. In the case of 3 non-drying oils the ability to form YP is not enhanced by oxidation, and only in one instance (olive oil) was it slightly increased. On the other hand, with the semi-drying and drying oils oxidation considerably and regularly enhanced this activity. These results were unexpected since oxidation increased the peroxide content of most oils regardless of whether they belong to the class of drying or non-drying oils. The degree of oxidation as measured by the peroxide numbers was irregular. Only in the case of castor oil did oxidation fail to increase the peroxide content.

Formation of YP and degree of peroxidation did not parallel each other. With the non-drying chaulmoogra oil for instance, the peroxide content increased more than 10-fold, although the ability to form YP remained almost unchanged. In the case of oleic acid the increase in peroxide content was less than 10-fold, but YP formation rose considerably. It was concluded from these observations therefore, that peroxide formation was not the essential factor responsible for the formation of YP by lipids. This hypothesis was confirmed by the results of experiments in which previously oxidized oils, upon heating at 165°C were found to retain considerable YP activity. The oils were heated in order to decrease the peroxide content to the original low level. Consequently, the increased production of YP following the oxidation of certain lipids is apparently due to oxidation products formed from or by the action of peroxides and not by the peroxides themselves. As already pointed out, Wilbur, Bernheim and Shapiro(12) arrived at the same conclusions in the case of thiobarbiturate reaction with unsaturated fatty acids.

Discussion. 1) The present study indicates that many bacterial, vegetable and animal lipids, as well as certain fatty acids, are capable of forming YP from PABA and PAS. Thus three different systems producing these YP are known, namely intact cells of mycobacteria and certain other micro-organisms, phenoloxidase, and lipids. The mechanism by which lipids are capable of forming YP was investigated in order that information might be gained as to the significance of PABA pigment formation and, at the same time, the possible physiological role of bacterial lipids. The results have suggested that the activity of lipids in transforming PABA is not a function of double bonds or of peroxides as such, as first supposed, but is associated with those oxidation products normally produced during rancidification or aging of lipids and fatty acids and which apparently are formed by and from peroxides. Is the formation of YP from aromatic amines by various bacterial lipids an incidental chemical property which has no physiological significance or is it biologically important? It is generally believed that the lipids or waxes of the tubercle bacillus act as a protective sheath against the defense systems of the host. The experiments reported upon in this paper suggest that lipids have a more active and vital role and participate, as with certain animal lipids, in the oxidoreductive cell metabolism as part of the general energy processes. Unsaturated lipids as well as their oxidation products are unstable in air and likely to set up redox systems between non-oxidized and oxidized forms. In this way, they may constitute a hitherto unrecognized oxido-reductive entity in addition to or supplementing the well-known oxido-reductive enzymes of protein nature.

2) The chemical nature of the PABA and PAS pigment formed by lipids is unknown. Earlier studies have suggested that the YP formed from PABA by actively growing mycobacterial cells contains quinoid oxidation products of PABA. Sloane, Fields and Mayer (13) found that highly purified PABA YP had a molecular weight of over 1000000 and contained, in addition to 2 aromatic nitrogenous components, at least 13 different amino acids. It is presumed that the yellow compound formed from PABA by lipids in the absence of proteins has a simpler chemical composition and lends itself to a simpler analysis than the yellow pigments formed in cultures of *M. tuberculosis* or *M. smegmatis*. According to Bernheim, Wilbur and Fitzgerald (14) PABA may condense with aldehydes or ketones to form the YP. On the other hand it is possible that an azo dye is formed from PABA which accounts for the bright and stable color of the YP.

3) The semi-micro filter paper method developed for the study of the YP formed by lipids seems applicable to the elucidation of many oxidative processes. It has the same advantages of application to chemical and enzymatic reactions as the filter paper method developed for the study of the water soluble system of phenoloxidase and other water soluble oxido-reductive reactions(11). Indeed both permit the use of very small amounts of reagents and the observation of many sequential reactions in the course of which colored intermediates or end products are formed, *viz.* melanin and indophenol blue formation, photobiological processes, etc. This method is also useful for the study of reactions during which colorless intermediates or end products are formed which are identified by color reactions such as proteinolysis, carbohydrate metabolism, fat oxidation, etc. A further advantage of this method is the possibility of preventing or interrupting the chemical processes, enzymatic or otherwise, at any stage of development by specific poisons applied during or before color development.

Summary. 1) As in the case of animal lipids and unsaturated fatty acids, various bacterial and vegetable lipids are capable of forming yellow pigment from p-aminobenzoic acid and p-aminosalicylic acid. 2) Oxidation considerably increases the activity of certain lipids to form the pigment. This enhanced activity is apparently not due to an increase in the peroxide content but rather to the formation of other oxidation products such as fatty aldehydes or ketones. 3) These results suggest that bacterial lipids play a role in the PABA pigment formation by growing cultures of mycobacteria. 4) The possible role of lipids in bacterial metabolism as important oxido-redox systems has been discussed. 5) A new filter paper method has been applied for the study of biologic oxidations of lipids and fatty acids.

---

1. Mayer, R. L., *Science,* 1943, v98, 203; *J. Bact.,* 1944, v48, 93, 337.
2. Mayer, R. L., and Crane, C., *Nature,* 1950, v165, 37.
3. Neumann, J., and Lavollay, J., *Compt. rend. Acad.,* 1948, v227, 373.
4. Spink, W. W., and Vivino, T. T., *Science,* 1943, v8, 44.
5. Sciarini, L. J., and Nord, F. F., *Arch. Biochem.,* 1945, v7, 367.
6. Cavill, G. W. K., and Vincent, J. M., *Nature,* 1945, v155, 301.
7. Reid, E. B., Pritchett, E. G., and Cushing, J. E., *J. Biol. Chem.,* 1952, v199, 443.
8. Kohn, H. J., and Liversedge, M., *J. Pharm. and Exp. Therap.,* 1944, v82, 292.
9. Bernheim, F., Bernheim, M. L. C., and Wilbur, K. M., *J. Biol. Chem.,* 1948, v174, 257.
10. Bloch, H., *J. Exp. Med.,* 1950, v91, 197.
11. Mayer, R. L., Grimm, M. R., and Kull, F. C., PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 163.
12. Wilbur, K. M., Bernheim, F., and Shapiro, O. W., *Arch. Biochem.,* 1949, v24, 305.
13. Sloane, N., Fields, D., and Mayer, R. L., unpublished data.
14. Bernheim, F., Wilbur, K. M., and Fitzgerald, D. B., *J. Gen. Physiol.,* 1947, v31, 195.

Received May 26, 1953. P.S.E.B.M., 1953, v83.

Effect of Soybean Phosphatide on Blood Coagulation Defect Following Total Body X-Irradiation in the Dog.* (20366)

SIDNEY G. WHITE,† JOHN B. LAGEN, PAUL M. AGGELER, AND ROBERT P. GEYER.
From the Department of Medicine, University of California School of Medicine, San Francisco, and the Department of Nutrition, Harvard University School of Public Health, Boston.

It has been reported that the active principle in platelets which contributes to the formation of thromboplastin from plasma sources resides in a phospholipid fraction of the platelets(1,2). The present investigation was conducted in order to determine whether soybean phosphatide can be used as a substitute for the platelet factor in dogs made thrombocytopenic by total body x-irradiation. Soybean phosphatide was chosen for this purpose because it is readily available and has been used previously to stabilize neutral fat emulsions and fat soluble vitamin K preparations for intravenous use in man(3).

Methods. Blood was obtained from the foreleg vein of the dog with one No. 18 gauge needle and 2 syringes coated with silicone oil.‡ Four and one-half ml of blood from the first syringe was added to 0.5 ml of 0.1 M potassium oxalate and used for plasma tests. The clotting time and prothrombin utilization tests were done on blood from the second syringe. *Whole blood clotting time (C.T.).* Two ml aliquots of blood were placed in each of two 13 by 100 mm glass tubes in a waterbath at 37°C. Starting at 3 minutes, both tubes were tipped every minute until each could be inverted without spilling the contents. The results were averaged. Incubation of the specimens at 37°C was continued until one hour had elapsed from the time the blood was drawn, at which time 0.22 ml of 0.1 M potassium oxalate was added to each tube and mixed with the clotted or clotting blood. The tubes were then promptly centrifuged to recover the serum for prothrombin testing. Clot retraction was observed at the end of one hour's incubation of the blood at 37°C. *Prothrombin assays on plasma and serum* were done by the 2-stage method of Ware and Seegers(4), using a lyophilized incubation mixture§ and bovine fibrinogen‖ in the test. A 1.25% solution of fibrinogen in 0.9% NaCl was adsorbed with freshly precipitated and washed barium sulfate in order to remove any traces of prothrombin. *Residual serum prothrombin determinations.* The number of units of prothrombin found in the serum was divided by the number of units of prothrombin found in the plasma and multiplied by 100 in order to determine the per cent residual serum prothrombin. A value of greater than 30% residual serum prothrombin is significant evidence of impaired prothrombin utilization. This test provides a sensitive index of the severity of the coagulation defect which occurs following total body x-irradiation(5-7). *In vitro assay of clotting properties of soybean phosphatide.* Two methods were employed: 1) 1.0 ml of blood was added to 0.1 ml of soybean phosphatide solution, and the effect on prothrombin utilization was determined. The results were compared with those produced by the addition of the blood to 0.1 ml of standardized tissue thromboplastin preparation.¶ 2) 0.1 ml of 0.025 M $CaCl_2$ was added to a mixture of 0.1 ml of dog plasma and 0.1 ml of soybean phosphatide solution in a 13 by 100 mm pyrex tube in a waterbath at 37°C. The clotting time of duplicate specimens was determined and the results averaged. The results were compared with those obtained when 0.1 ml of a standardized tissue thromboplastin preparation or 0.1 ml of 0.9% NaCl was substituted for the phosphatide solution in the test. *Platelet counts* were done by the method of Brecher and Cronkite(8). *Preparation of soybean phosphatide.* 400 g of soybean phosphatide** were dissolved in 1.6 liters of redistilled petroleum ether (B.P. 65°C) and filtered through an asbestos type bacterial filter. The filtrate was poured with rapid stirring into 4 liters of redistilled acetone and the precipitate collected on a filter paper. It was redissolved in petroleum ether and reprecipitated as before. The precipitate was washed well with acetone and freed of solvent by means of a stream of dry nitrogen. The product was a light tan-colored granular material, containing approximately 31% lecithin and 31% cephalin. The remainder was composed mainly of inositol phosphatides. For use in this study, the material was dissolved in 5% glucose in water to a concentration of 2.5%. *Technic of x-irradiation.*†† All dogs were given a total dose of 150 r of total body x-irradiation. This dose is LD_{50} in our laboratory. The irradiation was given in 2 doses of 75 r delivered to each side of the body, while the dogs were under light pentobarbital anesthesia. Radiation factors‡‡ were 250 KV constant potential, 12 ma, HVL 1.5 mm Cu; filter, 0.5 mm Cu and 1 mm Al. Target to skin distance (tsd) was 125 cm for all dogs. The mean mid-body dose rate of 14 r per minute was calculated for each dog individually by averaging equal numbers of Victoreen ionization-chamber readings taken on the proximal and distil skin surfaces at representative areas.

---

* Supported in part by a research grant from the National Cancer Institute, of the National Institutes of Health, Public Health Service, and The Adalynn Herndon Memorial Fund.

† Captain, MC, U. S. Air Force.

Opinions expressed are those solely of the authors and in no way reflect the official views of the U. S. Air Force.

‡ G. E. silicone oil No. 9996-300.

§ Difco Laboratories, Inc., Detroit, Mich.

‖ Armour Laboratories, Chicago, Ill.

¶ Simplastin—Chilcott Laboratories, Morris Plains, N. J.

Results. 1) *In vitro effects on prothrombin utilization.* The effect on prothrombin utilization of the addition of soybean phosphatide and of tissue thromboplastin to the blood *in vitro* is shown in Fig. 1. Blood obtained from a male dog, weighing 13 kg, 21 days after 150 r total body x-irradiation, in which no platelets were seen, was used in this experiment. The soybean phosphatide solution produced a more profound effect, which was less rapidly lost by dilution, than did the tissue thromboplastin. Neither substance induced clot retraction.

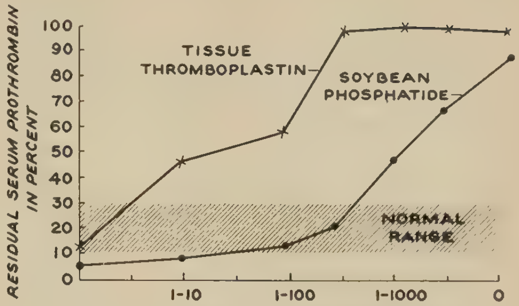


FIG. 1. Effect of addition of soybean phosphatide and of tissue thromboplastin on prothrombin utilization in the thrombocytopenic x-irradiated dog. (Serum tested after incubation one hr at 37°C of 1 ml of blood mixed with 0.1 ml of each dilution of substance tested. Prothrombin determinations by 2-stage method of Ware and Seegers(4).)

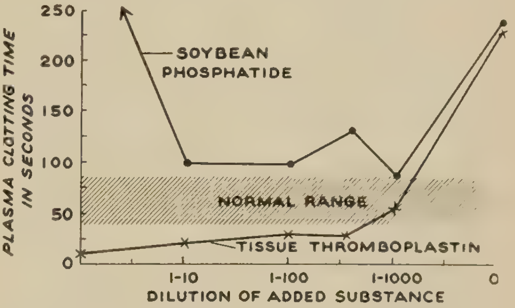


FIG. 2. Effect of addition of soybean phosphatide and of tissue thromboplastin on plasma clotting time of the thrombocytopenic x-irradiated dog. (One ml 0.025 M $CaCl_2$ added to 0.1 ml of plasma and 0.1 ml of substance tested at 37°C.)

2) *In vitro effects on plasma clotting time.* The effect on the plasma clotting time of the addition of soybean phosphatide and of tissue thromboplastin to the plasma of the same dog is shown in Fig. 2. The soybean phosphatide solution produced less shortening of the clotting time than did the tissue thromboplastin. In concentrated solution it proved to have anticoagulant properties. In this type of test the clot-promoting properties are apparently obscured by a clotting inhibitor in the phosphatide solution.

3) *Effect of slow intravenous infusion.* A male dog, weighing 13 kg, under light pentobarbital anesthesia, 8 days after 150 r total

---

** Grade "RG" soybean lecithin, Glidden Co., Chicago, Ill.

†† Authors are indebted to Dr. R. F. Kallman and Dr. Henry I. Kohn, Radiation Laboratory, University of California School of Medicine, for the x-irradiation of these animals.

‡‡ Westinghouse Quadroncondex Machine.

TABLE I. Effect of Intravenous Infusion during One Hour of 37 ml of 2.5% Soybean Phosphatide Solution (Diluted to 150 ml with 5% Glucose) on Blood Coagulation Defect in the Thrombocytopenic X-Irradiated Dog. No clot retraction in each experiment.

	Clotting time, min.	Platelet count/mm³ × 1000	Plasma prothrombin in units/ml*	Residual serum prothrombin in units/ml*	Residual serum prothrombin in % of plasma value
Before infusion	17	37	170	86	50
15 min. after infusion started	5		200	27	13
30 min. after infusion started	5	28.5	130	26	20
60 min. after infusion started (end of infusion)	<3		105	<10	<10
2 hr after end of infusion	7	38	140	23	16
22 hr after end of infusion	16	11	120	72	59

* Two-stage method of Ware and Seegers(4).

TABLE II. Effect of Intravenous Infusion during a Period of 25 Minutes of 100 ml of 2.5% Soybean Phosphatide Solution on Blood Coagulation Defect in the Thrombocytopenic X-Irradiated Dog. No clot retraction.

	Clotting time, min.	Platelet count/mm³ × 1000	Plasma prothrombin in units/ml*	Residual serum prothrombin in units/ml*	Residual serum prothrombin in % of plasma value
Before infusion	19	11.5	155	92	58
15 min.†	8	12	72	18	25
1½ hr†	14	10.5	90	45	50
3½ hr†	18		73	92	126

* Two-stage method of Ware and Seegers(4).
† After end of infusion.

body x-irradiation, whose blood showed 37000 platelets/mm³, no clot retraction, and a 17-minute C.T., was given 37 ml of the phosphatide solution diluted to 150 ml with 5% glucose in water by intravenous infusion in a period of 60 minutes. The results are shown in Table I. At the termination of the infusion, the C.T. was reduced to less than 3 minutes, and the residual serum prothrombin to less than 10%. Marked effects persisted for 2 hours, but had disappeared by 22 hours. A non-anesthetized female dog, weighing 11.5 kg, 11 days after 150 r total body x-irradiation, whose blood showed 11,500 platelets/mm³, no clot retraction, and a 19-minute C.T., was given 100 ml of the 2.5% phosphatide solution intravenously over a period of 25 minutes. The results are shown in Table II. Fifteen minutes after the infusion was completed, the C.T. was reduced to 8 minutes and the residual serum prothrombin to 25%. Both values had returned to the pre-infusion level at the end of 3½ hours. The platelet count and clot retraction remained unchanged. In both of these experiments the plasma prothrombin was also reduced by the infusion of the soybean phosphatide solution.

4) *Effect of small rapid intravenous infusion.* A male dog, weighing 13 kg, 9 days after 150 r total body x-irradiation, whose blood showed 11,500 platelets/mm³, no clot retraction, and a 14-minute clotting time, was given 20 ml of the 2.5% soybean phosphatide solution intravenously in a period of 3 minutes. The C.T. was reduced to 8 minutes during the observation period. There was no change in the platelet count or clot retraction. The effect on the residual serum

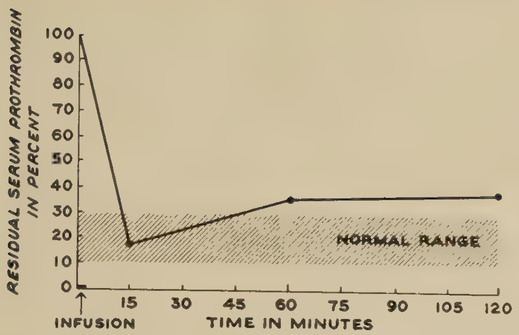


FIG. 3. Effect of rapid intravenous infusion of 20 ml of a 2.5% soybean phosphatide solution on prothrombin utilization in the thrombocytopenic x-irradiated dog. (Serum tested after incubation of blood for one hr at 37°C. Prothrombin determinations by 2-stage method of Ware and Seegers(4).)

prothrombin is shown in Fig. 3.

Summary. Thrombocytopenia, prolonged whole blood clotting time, and markedly impaired clot retraction and prothrombin utilization were induced in dogs by 150 r total body x-irradiation. A marked increase in prothrombin utilization was found after the addition of a soybean phosphatide solution to the blood *in vitro*. A temporary decrease in the clotting time and an increase in prothrombin utilization, without change in the platelet count or clot retraction, was induced by the intravenous administration of 20 to 100 ml of a 2.5% solution of the soybean phosphatide.

---

1. Milstone, J. H., *Gen. Physiol.*, 1951, v35, 67.

2. van Creveld, S., and Paulssen, M. M. P., *Lancet*, 1952, v1, 23.

3. Geyer, R. P., Mann, G. V., Young, J., Kenney, T. D., and Stare, F. J., *J. Lab. and Clin. Med.*, 1948, v33, 163.

4. Ware, A. G., and Seegers, W. H., *Am. J. Clin. Path.*, 1949, v19, 471.

5. Cronkite, E. P., Jacobs, G. J., Brecher, G, and Dillard, G., *Am. J. Roentgenol.*, 1951, v67, 796.

6. Jackson, D. P., Cronkite, E. P., Jacobs, G. J., and Behrens, C. F., *Am. J. Physiol.*, 1952, v169, 208.

7. Ferguson, J. H., Andrews, G. A., and Brucer, M., PROC. SOC. EXP. BIOL. AND MED., 1952, v80, 541.

8. Brecher, G., and Cronkite, E. P., *J. Appl. Physiol.*, 1950, v3, 365.

Received May 27, 1953. P.S.E.B.M., 1953, v83.

Effect of Glucagon in Stable and Unstable Diabetic Patients. (20367)

W. R. KIRTLEY, S. O. WAIFE, AND F. B. PECK. (Introduced by O. M. Helmer.)
From the Lilly Research Laboratories and Indianapolis General Hospital, Indianapolis, Ind.

A hyperglycemic factor, termed "glucagon" by Murlin *et al.*(1), has been known for 30 years to be present in most commercial insulin preparations. Its isolation, purification, and characterization has been an exceedingly stubborn problem. Recently, the authors have had available for study a preparation of an unusually high degree of purity provided by A. Staub of these Laboratories(2). The material used in this study was of such potency that 0.5 μg of the substance per kg administered intravenously to unfed cats anesthetized with amobarbital produced a rise in the blood sugar of 30 mg per 100 cc above normal within 25 minutes (a "cat unit"). Further animal assays showed it to contain only 0.005 unit of insulin per mg of solid.

During the course of an investigation of the physiological properties of this highly purified hyperglycemic-glycogenolytic factor (glucagon), the authors studied the response of various types of diabetic subjects. In this report only the response of the blood sugar and serum inorganic phosphorus will be discussed. Data relative to pyruvate, lactate, potassium, and certain steroid studies will be presented elsewhere(3).

Technic. An intravenous infusion of diluted glucagon was given both to normal persons and to 8 female diabetic patients. All subjects received 10 μg/kg body weight (10 μg of this material was equivalent to 20 "cat units") in the fasting state; the diabetics had not received insulin on the morning of the test. Since no dose exceeded 1 mg of the test material, no one received more than 0.005 unit of insulin based on animal assay results. Venous blood sugar was determined by the

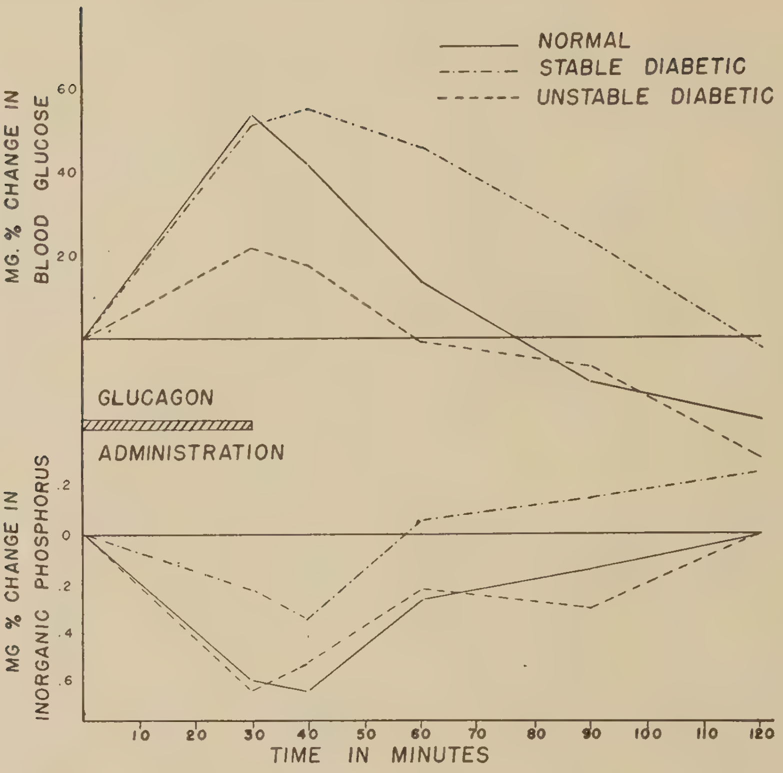


FIG. 1. The effect on blood sugar and inorganic phosphorus of intravenous glucagon in normal subjects and stable and unstable diabetic patients.

Nelson method; inorganic phosphorus, by the Fiske and Subbarrow technic adapted for a photoelectric colorimeter.

Results. The blood sugar levels of the normal presons reached a peak of elevation in about 10 minutes after completion of the infusion and fell to values below the fasting level between 60 and 90 minutes, much as is seen following glucose administration; however, diabetics receiving glucagon in a similar manner showed glycemic responses that could be divided into two patterns (Fig. 1).

In four patients the average blood sugar rose to a level closely approximating that of the normal subjects but fell slowly so that by 90 minutes it still exceeded the initial level, and by 120 minutes, in spite of individual variations, the average was about at the initial level. These patients also showed an impaired fall in serum inorganic phosphorus. The patients in this group seem to have in common the general clinical characteristics of the "stable, adult" type, that is, the middle-aged or elderly, overweight diabetic who is not particularly sensitive to insulin and is not liable to develop ketosis easily.

In 4 other diabetics, however, the blood sugar did not rise as high and fell below the initial value at 60 minutes. (The 60-minute point on the curve shows the differences between the two groups most clearly.) Whereas, at 120 minutes the blood sugar of the first group was at initial levels, it was 29.5 mg per 100 cc below in the second group. It was also noted that the glycemic response bore no relationship to the height of the initial glucose level. Quite in contrast with the other diabetics, these patients had a normal fall in serum phosphorus. Three of the patients in the second group were extremely labile showing spontaneous variations in blood glucose (when determinations were made 4 times daily) of as much as 300 mg per 100 cc or more in a 24-hour period, the insulin and diet remaining unchanged. They are in the category usually designated as being "unstable and insulin-sensitive."

Discussion. It was assumed that these unstable patients would have an even more abnormal response to endogenous glucose liberated by glucagon than the stable diabetics, in keeping with Bornstein's finding(4), that this type of patient is more likely to be deficient in circulating insulin. It is highly probable that the glycogen content of the liver is reflected in the height of the glucose elevation following glucagon administration. If it is assumed that the stable diabetic has endogenous insulin(4) and the unstable case has none, or very little, and that insulin increases glycogen deposition in the liver, it would appear that the labile diabetic with little or no circulating insulin has low hepatic glycogen stores. Glucagon would then produce a smaller immediate rise in blood sugar in such subjects. Contrariwise, the stable diabetic with his own circulating insulin and greater hepatic glycogen stores would show a normal response to the glycogenolytic factor.

The fall in blood sugar below initial fasting levels after glucose administration is considered by some to be the result of the stimulation of insulin. If this is so, then glucagon stimulated the production or activation of endogenous insulin in the unstable diabetic. This perhaps was mirrored in the normal fall in serum inorganic phosphorus in these patients. According to Forsham and Thorn(5), a fall in serum inorganic phosphorus (following intravenous glucose administration) is "a valid criterion for the peripheral utilization of glucose" and is said to be dependent on the presence of insulin(6-8).

Since the increment in blood sugar following glucagon was relatively small in unstable diabetics, insulin, if stimulated, was not called forth by the additional hyperglycemia, and some other mechanism must have been at work. Other explanations, of course, are possible, but there is no evidence at present to completely explain this phenomenon.

One might speculate that the stable type of diabetic is relatively insulin-insensitive, because he has glucagon to counteract any fall in blood sugar. He has some circulating insulin, but not enough for all metabolic needs, and he has ample liver glycogen. Such a diabetic would be comparable to the partially alloxanized animal. The unstable type of diabetic, on the other hand, may be insulin-sensitive, because he lacks endogenous glucagon needed to modify rapid falls in blood sugar. With little if any available insulin of his own, he may have a relative deficiency of liver glycogen. Such a diabetic would resemble the depancreatized animal.

Summary. A highly purified preparation of glucagon (pancreatic hyperglycemic-glycogenolytic factor, HGF) was administered intravenously to normal and diabetic subjects. Two different types of responses in blood sugar and serum inorganic phosphorus were found between the more common stable and the rarer labile diabetic. The responses seem to fall into patterns compatible with present concepts of the two broad clinical varieties of diabetes mellitus.

---

1. Murlin, J. R., Clough, H. D., Gibbs, C. B. F., and Stokes, A. M., *J. Biol. Chem.*, 1923, v56, 253.

2. Staub, A., Sinn, L. J., and Behrens, O. K., *Science*, 1953, v117, 628.

3. Kirtley, W. R., Waife, S. O., Peck, F. B., and Helmer, O. M., *Diabetes* in press.

4. Bornstein, J., and Lawrence, R. D., *Brit. M. J.*, 1951, v2, 1541.

5. Forsham, P. H., and Thorn, G. W., *Proc. Am. Diabetes A.*, 1949, v9, 101.

6. Bolliger, A., and Hartman, F. W., *J. Biol. Chem.*, 1925, v64, 91.

7. Soskin, S., Levine, R., and Hechter, O., *Am. J. Physiol.*, 1941, v134, 40.

8. DeVenanzi, F., *Rev. Soc. Argent. Biol.*, 1949, v25, 120.

Received May 25, 1953. P.S.E.B.M., 1953, v83.

Stimulating Effect of Nucleoprotein Fraction of Chick Embryo Extract on Homologous Heart Fibroblasts.* (20368)

R. J. Kutsky.† (Introduced by Morgan Harris.)

From the Department of Zoology, University of California, Berkeley.

Embryo extract as ordinarily prepared in balanced saline solution(1) stimulates growth in tissue cultures as first reported by Carrel (2). The experiments of Fischer(3) involving dialysis and ultracentrifugation suggest that the growth factors in embryo extract fall into two categories: (a) substances of high molecular weight, probably protein in nature, collectively termed "embryonin" by Fischer, and (b) small molecules readily diffusible through dialysis membranes. Several workers have attempted isolations of the active factors of high molecular weight. Fischer(4) using Hammersten's(5) technique isolated an active nucleoprotein fraction with several electrophoretic components of which 4 were predominant, but he did not test the biological activity of the components. Davidson and Waymouth(6) likewise isolated an active nucleoprotein fraction from chick embryos and Waymouth(7) reported a few electrophoretic peaks in a similar preparation, but their biological activity was not tested. Katsuta and Takaoka(8) claim that a high polymer pentosenucleic acid isolated from chick embryos by Nishioka and Ibuka(9) is Fischer's (4) "embryonin," the growth factor of high molecular weight in chick embryo extract.

Von Euler and Heller(10) have developed an isolation method for liver nucleoproteins by using streptomycin as a specific precipitant. A modification of this method designed for quantity isolation of a chick embryo nucleoprotein fraction will be described here. This fraction will be shown to contain most of the growth factors of high molecular weight in chick embryo extract. Preliminary characterizations of this fraction and tests on the high polymer pentosenucleic acid prepared according to Nishioka and Ibuka(9) will also be presented.

Isolation procedures. The nucleoprotein fraction was obtained from fresh 12-day chick embryos. These were removed from their membranes and cooled to 0°C. This temperature was maintained throughout the isolation procedure. The embryos were then homogenized in a large Potter homogenizer, stirred one hour with an equal volume of M/8 borate, pH 7.1, and centrifuged 15 minutes at 24,000 x g in a fluid-cooled Servall centrifuge. Re-extraction of the residue increased the final yield by about 30%. The supernatant was made M/100 in streptomycin sulfate (Lilly) by using a M/4 stock solution, pH 7.5, left undisturbed for 2 hours and the resulting precipitate centrifuged down at 24,000 x g for 10 minutes. The precipitate was gently homogenized with M/3 potassium phosphate buffer, pH 7.2, 4 cc per embryo, yielding a turbid solution which was dialyzed with stirring in ¾ inch Visking tubing for 12 hours against borate, pH 7.1, 120 cc per embryo. The streptomycin precipitation was repeated in the dialyzed solution with resuspension in phosphate as above. Dialysis with rotation of the sac was continued for 12 hours against two changes of M/3 phosphate buffer, 32 cc per embryo. The dense dialyzed suspension was then ultracentrifuged one hour at 105,000 x g in a Spinco refrigerated centrifuge. The free lipids rose to the surface and were removed with a capillary pipet leaving a clear, colorless solution containing soluble nucleoproteins above a pellet of cell particulates and insoluble materials. The solution was stir-dialyzed for 24 hours either against one change of distilled water, 120 cc per embryo equivalent prior to lyophilization or against the same volume of Gey's saline prior to culture operations. This solution, after ultra-

---

* Supported by grants to Dr. Morgan Harris from the Cancer Research Coordinating Committee, University of California. This work represents part of a thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, Department of Zoology, University of California.

† AEC Predoctoral Fellow in the Biological Sciences 1951-1953. Present address: Donner Laboratory of Medical Physics, Berkeley, Calif.

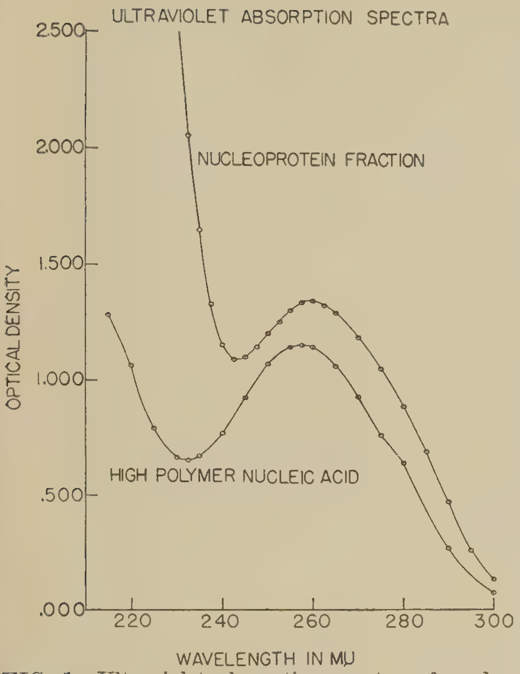


FIG. 1. Ultraviolet absorption spectra of nucleoprotein fraction and high polymer nucleic acid, both in Gey's saline (0.6 mg/cc) at pH 7.5.

centrifuging again for an hour at 105,000 x g, was clear, colorless, viscous and exhibited a typical nucleoprotein absorption spectrum in the ultraviolet with a maximum at 260 mμ and a minimum at 243 (Fig. 1). Concentration of the nucleoprotein fraction in the clear solution was routinely determined by comparing optical densities at 260 mμ against a concentration curve based on dry weight measurements. Duplicate analyses for PNA and DNA in the streptomycin-precipitated fraction are seen in Table I. For purposes of analysis, PNA and DNA were separated and protein removed by the method of Schmidt and Thannhauser(13) followed by Schneider's procedure(14). DNA was determined by the method of Stumpf(15), PNA by the orcinol method of Mejbaum(16). Direct preparation of high polymer pentose-nucleic acid from chick embryo extract followed the method of Nishioka and Ibuka(9). The ultraviolet absorption spectrum of the final product is shown in Fig. 1. The slight inflection at 280 mμ suggests residual protein. In addition, a preparation of nucleic acid was obtained from the streptomycin-precipitated nucleoprotein fraction according to Grinnan and Mosher (12) omitting the preliminary guanidine precipitation.

Tissue culture technic. Fresh explants of 13-16 day chick heart were cultivated in Carrel flasks at 37°C for a 10-day period. The basal assay medium consisted of a chicken plasma clot with a supernatant containing 40% horse serum and 60% Gey's saline. Supernatants were changed twice a week without washing the cultures. The media were not dialyzed. Details are given in a previous paper by Kutsky and Harris(11). The experimental cultures were supplemented with the nucleoprotein fraction in saline solution, each series being matched with a corresponding group of non-supplemented control cultures. The effectiveness of a particular fraction was judged by the increase in area of the culture at ten days, by the characteristic microscopic morphology of the cells in the culture and in certain cases by measurements of the pH levels in the supernatant fluids. Fractions were originally prepared by using sterile technics, but when this was omitted there was no sign of alteration in the final product since the temperature was maintained at 0°C throughout the isolation procedure. Fractions prepared under non-sterile conditions did not in general produce contaminations in tissue culture. Antibiotics were routinely incorporated in the media and the fractions were routinely subjected to one-hour's ultracentrifugation at 105,000 x g just prior to use to reduce contaminating microorganisms to a low level.

Results. Table II shows the relative increases in surface area, at 10 days, of cultures containing 3 concentrations of the nucleopro-

TABLE I. Nucleic Acid Determinations on Nucleoprotein Fraction.

Analysis	Sample wt, mg	Nucleic acid found, mg*	% nucleic acid	Avg % NA
PNA	10	1.05	10.5	10.3
	”	1.06	10.6	
	”	.99	9.9	
DNA	100	.33	.33	0.33
	”	.32	.32	

* Each value represents avg of two aliquot determinations.

TABLE II. Outgrowth of Chick Heart Fibroblasts in Undialyzed Media Supplemented with Fractions from Chick Embryo Extract.

Supplement to basal medium*	Areal increase 10 days mm²† Supplemented	Controls	Diff. from unsupplemented controls	Signif. of diff. P
Nucleoprotein fraction				
.2 mg/cc	34.7 ± 2.3	14.3 ± 1.4	20.4 ± 2.7	<.001
.4 mg/cc	53.4 ± .9	14.3 ± 1.4	39.1 ± 1.7	<.001
.7 mg/cc	65.0 ± 1.2	14.3 ± 1.4	50.7 ± 1.8	<.001
Supernatant dialyzed‡				
15%	22.8 ± 1.3	13.9 ± 1.3	8.9 ± 1.9	<.01
High polymer nucleic acid				
1 mg/cc	10 ± .6	13.8 ± 3.9	3.8 ± 4.0	>.3
Nucleic acid from nucleoprotein				
.3 mg/cc	17 ± 3	19.9 ± 4.6	2.9 ± 5.5	>.5

* Basal medium: Plasma clot, 40% horse serum, 60% Gey's solution.

† Mean value and stand. error (Snedecor(17)). Six cultures/series except last supplement where 3 used.

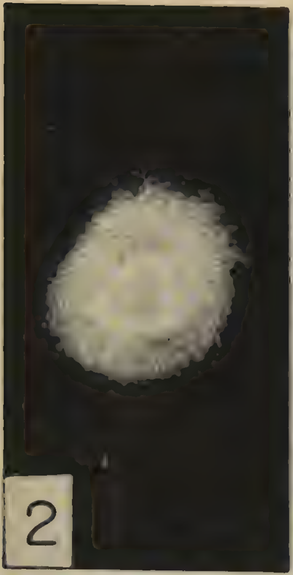
‡ From first streptomycin precipitation, replacing ¼ of Gey's solution in culture.

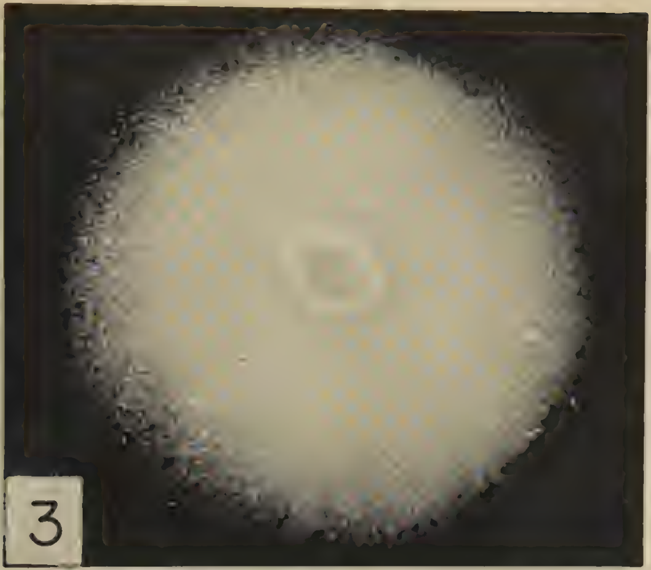
tein fraction as compared to outgrowth of similar cultures in the unsupplemented basal assay medium. The obvious increases in cultures containing the nucleoprotein fraction are further documented by the photomicrographs shown in Fig. 2, 3. Increases in surface area were also associated with characteristic alterations in cell morphology. In the unsupplemented control cultures the cells were heavily granulated and densely massed without conspicuous orientation (Fig. 4). In cultures containing the nucleoprotein fraction the outgrowth zone was much less granular, with the marginal cells broad and flat, tending toward a looser, more radial arrangement (Fig. 5). It should be emphasized that the basal-assay medium contained undialyzed chicken plasma and horse serum. Consequently it has not been possible to separate the relative role of nucleoprotein and dialyzable factors in the total effect obtained. Synergism between these factors has recently been demonstrated in dialyzed assay media by Harris and Kutsky(18) and will be described later in more detail.

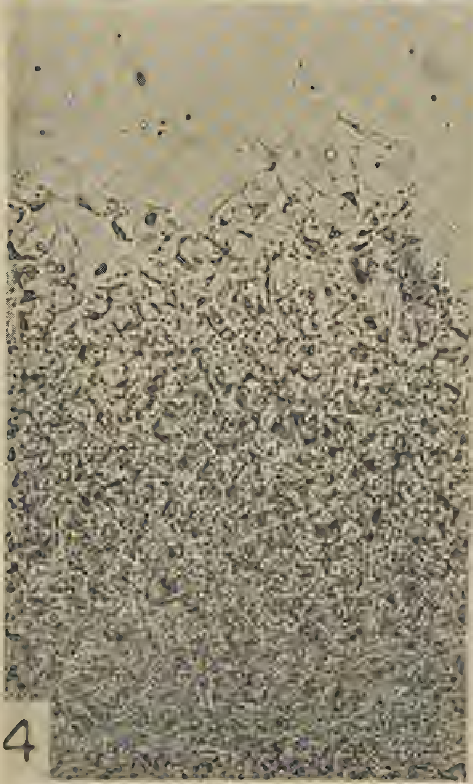
Table II also includes data on areal increases in cultures supplemented with the supernatant (thoroughly dialyzed against Gey's saline) from the first streptomycin precipitation. The supernatant fraction was incorporated at the level of 15% of the volume of the medium, replacing a like amount of Gey's saline. It is apparent that the residual activity in the supernatant is small in comparison to that of the streptomycin precipitate.

Assays of the high polymer PNA prepared according to the method of Nishioka and Ibuka(9) were uniformly negative as were similar experiments with nucleic acid isolated directly from the nucleoprotein fraction(12). Neither of these preparations produced increases in surface area (Table II) or alterations in cell morphology similar to those seen with the nucleoprotein fraction.

Discussion. The original isolation technic of von Euler and Heller(10) proved unsuitable for chick embryo extract and was therefore modified in the present experiments. Dialysis against distilled water to remove interfering salts as specified in the original procedure partially denatured the chick embryo proteins. Homogenization in and precipitation from borate, suggested by experiments of Gros and Rybak(19), eliminated the initial dialysis and doubled the effectiveness of the first streptomycin precipitation. The original streptomycin sulfate concentration of M/800 resulted in only a partial precipitation of chick embryo nucleoproteins, but when increased 8-fold to M/100 gave a maximum yield. In







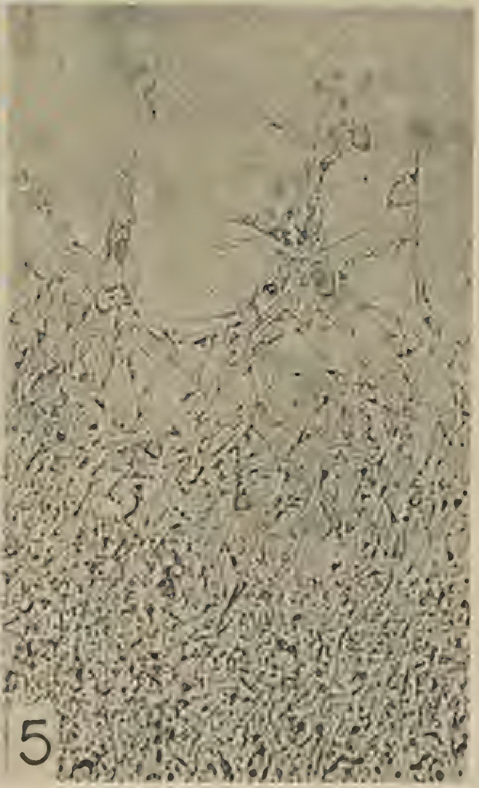


FIG. 2. Outgrowth of chick heart fibroblasts after 10 days in unsupplemented basal assay medium. × 7½.

FIG. 3. Outgrowth at 10 days in basal medium supplemented with nucleoprotein fraction in Gey's saline. × 7½.

FIG. 4. Cellular detail at 10 days in culture of chick heart fibroblasts in unsupplemented basal medium. × 122.

FIG. 5. Similar view of culture containing supplementary nucleoprotein fraction in Gey's saline. × 122.

general, owing to the extremely fine precipitates formed by embryonic nucleoproteins, much higher centrifugal speeds were necessary than in the original method. The small residue of activity remaining in the supernatant after the first streptomycin precipitation may be due to the gradual nature of the precipitation of the chick nucleoprotein in contrast to the liver nucleoprotein. On standing, this precipitate develops a coarse texture which sediments at lower speeds and fortunately occludes less heme-protein than the initial fine precipitate. The presence of contaminating heme-proteins was followed at their absorption maximum at 405 mμ. Development of the coarse texture made it unnecessary to wash the precipitate and eliminated the third and fourth reprecipitations of von Euler and Heller's method which resulted in heavy losses. Trials with various salts revealed that substitution of M/3 potassium phosphate for M sodium chloride to break up the streptomycin-nucleoprotein complex and to dissolve the nucleoprotein fraction reduced the large volumes of solvent originally required. Residual streptomycin associated with the nucleoprotein fraction could be split off by dialyzing against the phosphate and was checked by determining the increase in optical density at the streptomycin maximum at 325 mμ after incubation with alkali. The streptomycin when bound to the nucleoprotein loses its characteristic absorption maximum at 325 mμ. Von Euler and Heller's procedure made no provision for removal of the free lipids or the microsomes, whereas they are here removed by ultracentrifugation. Lyophilization preserves most of the activity of the redissolved nucleoprotein fraction but the material is slow to redissolve, about 30% remaining insoluble. The material retains its biological activity in the dissolved state for 3 weeks when kept in the refrigerator.

To check the possibility that streptomycin itself may have a stimulating effect in tissue culture, various concentrations (M/40 to M/4000) were tested in culture in the same manner as was the nucleoprotein fraction. None of the concentrations were stimulating, indeed the higher ones were inhibitory. A similar inhibition was recently reported by Fusillo, Metzger, and Kuhns(20).

High polymer pentosenucleic acid (relative viscosity 2.4) isolated according to Nishioka and Ibuka(9) had no effect in tissue culture at 1 mg/cc and although biuret negative, showed a definite positive Sakaguchi test for protein. (See also slight inflection at 280 mμ, Fig. 1). Similar material was also tested in cultures in the absence of horse serum at a level of .3 mg/cc with embryo extract present, likewise with no positive results. It is possible that the positive results obtained by Katsuta and Takaoka(8) could be explained by a larger concentration of the contaminating active nucleoprotein fraction in their preparations than in the present experiments where more sensitive protein tests were used. The nucleic acid isolated from the nucleoprotein fraction by the method of Grinnan and Mosher(12) also showed no activity (Table II).

A preliminary electrophoretic separation was performed on an early preparation of the nucleoprotein fraction through the courtesy of Dr. R. Trautman of the Donner Laboratory of Medical Physics. This separation resulted in 4 subfractions of which only the major component was active when tested in culture. This component had an ultraviolet absorption spectrum similar to a nucleoprotein. Culture tests of the minor subfractions showed no activity but these may have been too dilute to show an effect. Two of the minor subfractions resembled nucleic acids in their ultraviolet absorption spectra while the third subfraction gave no spectral evidence of an absorption maximum at 260 mμ.

The small residual activity in the supernatant from the first streptomycin precipitation indicates that the nucleoprotein fraction contains most of the outgrowth-promoting substances of high molecular weight in chick embryo extract. The concentration series shows that a relatively large amount (0.7 mg/cc) is required to elicit an optimal effect.

A yield of approximately 2 g of lyophilized material has been obtained from 130 embryos. The nucleoprotein fraction gives positive biuret and Sakaguchi tests for protein. Analyses for nucleic acids (Table I) revealed that 0.3% DNA and 10.3% PNA were present in 2 samples of lyophilized nucleoprotein fraction. The DNA is probably an inactive contaminant in the procedure, since culture tests on both low and high polymer DNA from various sources showed no stimulating effect.

Summary. 1. A quantity isolation method is described for a chick embryo nucleoprotein fraction based on von Euler and Heller's(10) procedure for liver nucleoproteins. 2. When this fraction was tested in an assay medium of undialyzd chicken plasma and dilute undialyzed horse serum large outgrowths of chick heart fibroblasts were obtained with well-defined alterations in cell morphology and mode of outgrowth. 3. The nucleoprotein fraction described contains protein, 10.3% PNA, 0.3% DNA and has a characteristic nucleoprotein absorption spectrum. 4. High polymer pentose nucleic acid isolated according to Nishioka and Ibuka(9) produced no changes in size or appearance of assay cultures.

I am greatly indebted to Dr. Morgan Harris for helpful advice as well as for the provision of tissue culture and other facilities used in the research, to Dr. D. Mazia for consultation, use of the Spinco ultracentrifuge, lyophil apparatus, and samples of high polymer DNA, and to Miss C. Brode, Mr. L. Resseguie and Mr. D. Farquhar for technical assistance in tissue culture operations. A generous gift of streptomycin sulfate was made by the Eli Lilly Co. The photomicrographs used in this paper were taken by Dr. Harris and Mr. Victor Duran.

---

1. Parker, R. C., *Methods of Tissue Culture,* Hoeber, New York, 1950.

2. Carrel, A., *J. Exp. Med.,* 1913, v17, 14.

3. Fischer, A., *Biology of Tissue Cells,* Hafner, New York, 1946.

4. Fischer, A., and Astrup, T., *Arch. ges. Physiol.,* 1943, v247, 34.

5. Hammersten, E., *Z. physiol. Chem.,* 1920, v109, 141.

6. Davidson, J. N., and Waymouth, C., *Biochem. J.,* 1945, v39, 188.

7. Waymouth, C., PROC. SOC. EXP. BIOL. AND MED., 1947, v64, 25.

8. Katsuta, H., and Takaoka, T., *Jap. J. Exp. Med.,* 1952, v22, 163.

9. Nishioka, K., and Ibuka, K., *Jap. J. Exp. Med.,* 1952, v22, 163.

10. Von Euler, H., and Heller, L., *Arkiv Kemi,* 1948, v26A, No. 10, 1.

11. Kutsky, R. J., and Harris, M., *J. Cell. Comp. Phys.,* in press.

12. Grinnan, E. L., and Mosher, W. A., *J. Biol. Chem.,* 1951, v191, 719.

13. Schmidt, G., and Thannhauser, S. J., *J. Biol. Chem.,* 1945, v161, 83.

14. Schneider, W. C., *J. Biol. Chem.,* 1945, v161, 293.

15. Stumpf, P., *J. Biol. Chem.,* 1947, v169, 367.

16. Mejbaum, W., *Ztschr. physiol. Chem.,* 1939, v258, 117.

17. Snedecor, G. W., *Statistical Methods,* Iowa State College Press, Ames, Iowa, 1950.

18. Harris, M., and Kutsky, R. J., *Anat. Rec.,* 1953, v115, 6.

19. Gros, F., and Rybak, B., *Helv. Chim. Acta,* 1948, v31, 1855.

20. Fusillo, M. H., Metzger, J. F., and Kuhns, D. N., PROC. SOC. EXP. BIOL. AND MED., 1952, v79, 377.

Received April 30, 1953. P.S.E.B.M., 1953, v83.

L-Lyxoflavin in the Starting Diets of Turkeys and Chickens.[*] (20369)

D. H. SHERWOOD,[†] E. G. HILL, AND H. J. SLOAN. (Introduced by G. M. Briggs.)

From the Department of Poultry Husbandry,[‡] University of Minnesota, St. Paul.

Since Pallares and Garza(1) described the isolation of L-lyxoflavin there has been considerable interest in this compound in nutrition although there is still comparatively little published information concerning its essentiality in the diet. Emerson and Folkers(2),

---

[*] Supported in part by the Northwest Feed Manufacturers Assn., Minneapolis, Minn. and by Merck and Co., Rahway, N. J.

[†] Present address: Larro Research Laboratories, Detroit, Mich.

[‡] Publication No. 2909, Scientific Journal Series of the Minn. Agric. Station.

working with hyperthyroid rats, reported that L-lyxoflavin had a growth promoting activity distinct from that obtained with various liver fractions. Wahlstrom and Johnson(3,4) used L-lyxoflavin in a synthetic milk for pigs and reported highly significant growth increases at 7 weeks. Bruins *et al.*(5) obtained a growth increase of about 10% when L-lyxoflavin was fed to chicks for an experimental period of 2 weeks. Their diet was of a purified type and contained riboflavin. Cooperman *et al.* (6), using the ration described by Bruins *et al.*(5), obtained a small response from feeding supplemental L-lyxoflavin, but no more than was obtained by the addition of supplemental riboflavin at equivalent levels. Shorb (7) reported that L-lyxoflavin inhibited riboflavin competitively in the growth of *L. casei* in a riboflavin assay medium, but at high levels of riboflavin the L-lyxoflavin stimulated the growth of the organism. Ershoff(8) has reported that L-lyxoflavin is ineffective as an antithyrotoxic factor for rats although various liver substances were effective.

Because of the conflicting results with this compound reported in the literature, we are presenting our studies. No previous results with turkeys have been reported. A number of experiments have been conducted in this laboratory using L-lyxoflavin in experimental diets for both turkeys and chickens.

Experimental methods. All experiments were started with day-old poults or chicks and conducted for a period of approximately 4 weeks. Each experimental group consisted of 10 birds raised in standard electrically-heated batteries, with feed and water being supplied *ad libitum*. The turkey poults used were Broad Breasted Bronze of medium fast-growing strains. The ration used in the turkey work was a corn-soybean oil meal type ration described by Sherwood and Briggs(9) modified by the addition of 0.1% choline chloride, 10 μg vit. B_{12} and 10 mg procaine penicillin G per kg of ration. This ration calculates to be adequate in all other B-complex vitamins. In view of the recent report by Cooperman *et al.*(6) data from a separate experiment are shown in Table I which indicate that the basal ration was not improved by the inclusion of additional riboflavin or riboflavin rich materials. The purified diet used in the chick trials was as follows: 59.2% glucose, 25% Alpha protein,[§] 4% clarified corn oil, 6% salts 3M,[||] 5% cellulose, 0.6% DL-methionine, 0.2% choline chloride and the following vitamins (in mg/kg): thiamine hydrochloride, 20; riboflavin, 8; calcium pantothenate, 20; pyridoxine hydrochloride, 6; biotin, 0.2; nicotinic acid, 100; folic acid, 2; vit. B_{12}, 0.01; alpha tocopherol acetate, 10; menadione, 1. Vit. A was added at the rate of 20,000 IU/kg; vit. D_3 750 ICU/kg; and procaine penicillin G, 15 mg/kg. The chicks used were New Hampshire birds of a medium fast-growing strain. Mortality in all groups was negligible. The L-lyxoflavin used was crystalline L-lyxoflavin obtained from Dr. Karl Folkers, Merck & Co., Inc., Rahway, N. J. We also used in one trial a concentrate containing 33 mg L-lyxoflavin/g.

TABLE I. Effect of Addition of Other Vitamins on 4 Week Weights of Turkeys.

Exp. No.	Supplement added	Sex	No. birds/ mortality	28 day wt
1	0	—	10/0	575
	Dried brewers yeast, 5%	—	20/0	564
2	0	♂	6/0	560
	Niacin, 40 mg/kg	♂	6/0	567
	Riboflavin, 25 mg/kg	♂	6/0	555

Results and discussion. The results of the experiments with turkeys are shown in Table II. In the first 2 experiments, in which only male birds were used, the differences in growth favored the poults fed the L-lyxoflavin-supplemented diet by a highly significant margin. The percentage increase resulting from the use of L-lyxoflavin was 12.0% and 17.6% in Exp. 1 and 2, respectively. In the third trial, in which poults of mixed sexes were used, the difference was 7.1%, but this difference was not significant at the 5% level. It is believed

---

[§] Alpha protein, an isolated soybean protein manufactured by The Glidden Co., Chicago, Ill.

[||] Salts 2M (Hill, E. G., and Briggs, G. M., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 417) modified by replacing the ferric ammonium citrate with ferrous ammonium sulfate to give equivalent amounts of iron.

TABLE II. Growth Response of Poults Resulting from Adding L-Lyxoflavin to a Corn-Soybean Ration.

Exp. No.	L-lyxoflavin added, mg/kg	Sex	No. birds/ mortality	27 day wt (g)	Stand. error	F. E.*
1	0	♂	10/1	546	± 16.6	2.04
	25	♂	”	612†	± 17.4	1.79
2	0	♂	10/2	535	± 34.9	1.94
	35	♂	10/0	629†	± 20.8	1.84
3	0	♀ + ♂	”	537		1.92
	0	♀ + ♂	”	532	± 14.9§	1.95
	33‡	♀ + ♂	10/1	583		1.98
	33‡	♀ + ♂	11/1	572	± 16.5§	2.10

* F. E. = feed efficiency, or $\frac{\text{g feed consumed}}{\text{net g gained}}$.

† Wt increase compared to control, significant at 1% level by "t" test.

‡ Lyxoflavin added in form of concentrate.

§ Duplicate groups considered together.

TABLE III. Growth Response of Chicks Fed a Purified Diet Supplemented with L-Lyxoflavin. 13 experiments.

Series No.*	43		45		46		48	
Sex	♂♂		♀ + ♂		♀ + ♂		♂♂	
Supplement to basal	4 wk wt, g	S.E.†	4 wk wt, g	S.E.†	4 wk wt, g	S.E.†	4 wk wt, g	S.E.†
Basal alone	295	± 7.2	335	± 12.1	317	± 9.5	329	± 11.0
25 mg L-lyxoflavin/kg	306	± 6.6	331	± 7.2				
10% soybean oil meal	335	± 10.7	338	± 10.0				
Basal minus cellulose + 10% soybean oil meal			350	± 13.1				
As gp. 4 + 25 mg L-lyxoflavin/kg			360	± 10.5				
10% dried whey product + 25 mg L-lyxoflavin/kg					330	± 12.9		
10% torula yeast + 25 mg L-lyxoflavin/kg					324	± 10.6		
10% whey product + 10% torula yeast					335	± 7.4		
As gp. 8 + 25 mg L-lyxoflavin/kg					341	± 10.6		
5% torula yeast							333	± 9.7
As gp. 10 + 25 mg L-lyxoflavin/kg							342	± 8.4
As gp. 10 + 10 mg % orotic acid							349	± 6.8
As gp. 12 + 25 mg L-lyxoflavin/kg							336	± 8.3

* 20 New Hampshire chicks per group.

† Stand. error of the mean.

that the smaller response in the third experiment was due to the use of birds of mixed sexes, as females do not usually grow as fast as males.

Feed efficiency was slightly improved when L-lyxoflavin was added to the diets of male poults, but this was not the case when poults of mixed sexes were used.

The results of the trials with male chicks are shown in Table III. In no case did the addition of L-lyxoflavin result in any growth improvement that could be considered significant. In this respect we have failed to corroborate the findings of Bruins *et al.*(5) even though the diet used in these experiments was similar. It will be noted that orotic acid also failed to give any growth response with this diet. Further, the combination of orotic acid, L-lyxoflavin and dried yeast failed to result in any growth response over the dried yeast alone or the dried yeast plus the L-lyxoflavin. Similarly in trials not presented in Table III no growth response was noted with 20 chicks fed a corn-soybean diet supplemented with L-lyxoflavin, crystalline riboflavin, vit. B_{12} and procaine penicillin G in 2 trials.

No explanation can be offered as yet for the small growth stimulating action of L-lyxoflavin. Emerson and Fokers(2) suggested that it was of vitamin nature; but before this compound can be accepted as a vitamin, evidence for its existence in natural foods must be found. Before the growth-promoting effect of L-lyxoflavin can be considered of practical importance, studies must be made with this compound using diets of a more varied nature including a wider range of natural feed ingredients. Studies of this nature are at present in progress.

Summary. In trials with turkey poults highly significant growth responses were obtained with male poults fed a corn-soybean diet supplemented with L-lyxoflavin. Chicks fed purified diets failed to show a growth response when the diet was supplemented with L-lyxoflavin.

We wish to thank Merck and Co., Inc. for the L-lyxoflavin and crystalline vitamins, Lederle Laboratories for crystalline folic acid, and Professor T. H. Canfield for sexing the birds.

1. Pallares, E. S., and Garza, H. M., *Arch. Biochem.*, 1949, v22, 63.

2. Emerson, Gladys A., and Folkers, K., *J. Am. Chem. Soc.*, 1951, v73, 5383.

3. Wahlstrom, R. C., and Johnson, B. C., *J. Animal Sci.*, 1951, v10, 1065.

4. ———, Proc. Soc. Exp. Biol. and Med., 1952, v79, 636.

5. Bruins, H. W., Sunde, M. L., Cravens, W. W., and Snell, E. E., Proc. Soc. Exp. Biol. and Med., 1951, v78, 535.

6. Cooperman, J. M., Marusich, W. L., Scheiner, J., Drekter, L., DeRitter, E., and Rubin, S. H., Proc. Soc. Exp. Biol. and Med., 1952, v81, 57.

7. Shorb, Mary S., Proc. Soc. Exp. Biol. and Med., 1952, v79, 611.

8. Ershoff, B. H., Proc. Soc. Exp. Biol. and Med., 1952, v79, 469.

9. Sherwood, D. H., and Briggs, G. M., *Poultry Sci.*, 1951, v30, 902.

Received December 8, 1952. P.S.E.B.M., 1953, v83.

Distribution of P^{32} Following the Injection of TEPA* in Rats.† (20370)

M. L. Crossley, J. B. Allison, R. Wannemacher, J. Migliarese, R. P. Parker, E. Kuh, D. R. Seeger, and R. Partridge.

From the Bureau of Biological Research, Rutgers University, New Brunswick, N. J., and The Calco Chemical Division of the American Cyanamid Company, Bound Brook, N. J.

The ethylenimines have been shown to be effective in producing regressions of certain transplantable tumors in rats(1-3). These compounds will inhibit growth and cause cellular destruction of the tumor tissue at concentrations which will not produce any demonstrable changes in normal tissue. At higher concentrations, however, they will inhibit the growth of the testes and white blood cells(2,3). Still higher concentrations will cause general tissue damage. Studies have been started, therefore, to determine the specific effects of these compounds upon the various tissues together with their distribution throughout the body. A preliminary report is presented here of the distribution in rats of P^{32} from N, N′, N″-triethylenephosphoramide (TEPA*).

Materials and methods. Fifty-eight Sprague-Dawley male rats (175-200 g) were divided into 3 groups, 2 of which were implanted subcutaneously with the Flexner-Jobling carcinoma, the third group serving as a control. Ten days after transplantation, each of 24 rats in Group A bearing the tumors received one dose subcutaneously of TEPA*, 5.0 mg/rat, (900 μg P/rat), (1.55×10^4 CPM/μg P). In Group B the 16 control rats were treated with TEPA* at the same dosage level as the rats in Group A. The 18 rats in Group C bearing the tumors were injected subcutaneously with Na_2HPO_4*, 4.3 mg/rat, (400 μg P/rat), (7.16×10^3 CPM/μg P). At 1, 2, 15, and 30 hours following injection of the tagged compound, several animals from each group were autopsied. Samples of liver,

---

* Radioactive.

† Supported in part by a grant-in-aid from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council and by the Calco Chemical Division of the American Cyanamid Co.

TABLE I. Distribution of P^{32} after Injection of TEPA* or Na_2HPO_4* into Rats. Each average represents data from 6 animals in the tumor groups and four in the control groups.

	Interval after injection											
	1 hr			2 hr			15 hr			30 hr		
	µg P^{32} per g of wet tissue											
Group	Avg	Max	Min	Avg	Max	Min	Avg	Max	Min	Avg	Max	Min
TEPA*-Control												
Liver	3.3	4.6	2.4	3.0	3.5	2.0	2.9	3.0	2.8	2.5	3.0	1.7
Testis	3.6	4.6	1.9	4.0	4.7	3.7	1.4	1.7	1.2	1.0	1.2	0.7
Kidney	4.8	6.1	3.3	4.9	6.4	3.5	1.3	1.4	1.2	1.2	1.4	1.1
Spleen	2.4	3.3	1.8	2.7	2.9	2.5	4.8	6.1	4.1	7.0	8.3	5.8
Bone	1.9	2.3	1.0	1.9	2.3	1.8	4.5	4.8	4.0	4.8	6.2	3.5
TEPA*-Tumor												
Liver	3.6	4.8	2.5	2.9	4.3	2.0	2.6	3.3	2.0	2.6	3.1	2.0
Testis	2.5	4.9	0.5	3.1	5.1	0.6	1.1	1.5	0.6	0.9	1.1	0.7
Kidney	5.5	8.1	4.1	6.3	14.4	1.4	2.3	2.8	1.6	1.6	2.6	1.0
Spleen	2.5	4.3	0.4	2.8	3.9	0.8	2.8	3.4	2.1	2.9	4.7	1.9
Bone	2.5	5.7	1.3	2.2	3.3	1.0	4.5	5.4	3.9	5.3	6.0	4.7
Tumor	2.7	4.0	0.5	2.4	4.1	1.2	2.1	2.4	1.8	3.3	4.8	2.1
Na_2HPO_4*-Tumor												
Liver	8.3	10.0	7.4	6.7	10.0	3.8	3.0	4.2	2.4			
Testis	.4	.5	.3	.4	.7	.1	.1	.3	0			
Kidney	10.0	11.1	6.0	4.4	5.5	3.7	1.8	2.0	1.6			
Spleen	4.3	5.6	3.7	2.9	3.6	2.3	2.3	3.0	1.8			
Bone	9.4	11.0	5.1	13.9	18.7	9.7	13.2	15.5	12.1			
Tumor	4.9	5.9	4.6	3.0	4.2	3.3	2.5	3.0	2.0			

* Radioactive.

kidney, testes, spleen, bone, and tumor were taken for assay. Weighed samples of each tissue were digested in a mixture of HNO_3 and H_2SO_4, 1:1, and 100 λ aliquots of the digest were counted with an end window (1.4 mg/cm²) GM counter. Urine and fecal samples were collected from 6 rats of Group A and 4 rats of Group B for 24 hours following injection of the tracer. The samples were pooled within each group and prepared for counting as described above. Blood samples were obtained at the 2-hour interval from 6 rats in Group A and 4 rats in Group B and pooled within each group. Both plasma and serum were prepared and fractioned as follows. The globulins were precipitated by ½ saturation with $(NH_4)_2SO_4$. The remaining proteins were precipitated, first with sulfosalicylic acid and then phosphotungstic acid. P^{32} activities were determined on these 3 resultant filtrates as well as on the original serum and plasma. Samples of the liver, testes, and tumor obtained from Groups A and B at the 2-hour interval were fractioned for cellular components according to Schneider and Hogeboom(4). To do this, tissue was homogenized with ice cold (0.25 M) sucrose solution. The homogenate was centrifuged for 10 minutes at 700 x gravity, a procedure that separates nuclei from cellular components in liver tissue. Similarly, the mitochondria of liver cells were separated by centrifugation at 5000 x gravity and the microsomes at 25,000 x gravity. Since cellular particles have not been studied in detail in the testis and tumor tissues, the particles separated by this procedure will be characterized in terms of gravity.

Results. Distribution in tissues. The distribution of P^{32} following injection of tagged TEPA* or Na_2HPO_4* in various tissues of the rat is recorded in Table I. These data illustrate the rather uniform distribution of P^{32} activity from TEPA* throughout the body including the tumor. However, the P^{32} following injection of TEPA* concentrated more rapidly in the testes and less rapidly in bone than following injection of inorganic phosphate. There was a marked increase in activity in the spleen over a period of 29 hours (2.4 to 7.0 µg P^{32}/g spleen) in normals receiving TEPA*. The average size of the spleen was 300 mg/100 g body weight making an average of 2100 µg of P^{32} in the entire spleen

TABLE II. Distribution of P^{32} following Injection of TEPA* in Cellular Fractions in % of Total Activity. Particle size recorded in terms of gravity used for centrifugation.

Animal	Tissue	700 × gravity	5000 × gravity	25000 × gravity	Soluble
Normal	Liver	20.0	4.8	6.7	68.5
Tumor-bearing	"	18.3	5.8	5.4	70.1
Normal	Testis	22.2	3.2	4.2	70.5
Tumor-bearing	"	27.4	2.5	2.1	68.0
"	Tumor	15.2	3.8	4.3	76.5

* Radioactive.

30 hours following injection of TEPA*. The µg of P^{32}/g of spleen on the other hand, remained essentially constant in tumor-bearing animals. The size of the spleen, however, was increased in these animals to 900 mg/100 g body weight making the total activity at the end of 30 hours 2610 µg P^{32}, almost identical with the normal spleens. Concentration of P^{32} from TEPA* in the spleen may be associated with specific effects on the reticuloendothelial system.

Distribution in the cells. One type of measurement of the distribution of P^{32} from TEPA* in liver, testes, and tumor cells is illustrated in Table II. These data indicate a combination of P^{32} with the nuclei and/or other relatively heavy cell components as separated by centrifugation at 700 x gravity. Very little activity was found in lighter particles separated by 5000 or by 25,000 x gravity. Approximately 70% of the activity remained in the soluble fraction not thrown out by centrifugation. This soluble P^{32} remained in the filtrate after precipitating proteins with phosphotungstic acid.

Distribution in plasma. Two hours after injection of TEPA*, tagged with P^{32}, blood samples were taken from control and tumor-bearing rats. Approximately 60% of the P^{32} activity of either serum or plasma was precipitated from solution with the globulin fraction. The remaining 40% was in solution even after precipitating the proteins and polypeptides with sulfosalicylic acid and phosphotungstic acid. These results together with those presented previously suggest that TEPA* can combine with certain proteins, possibly thereby inhibiting some metabolic pathways.

Excretion. The activity in the excreta collected over a period of 24 hours following the injection of TEPA* was identical in normal and tumor-bearing animals. In both groups, 62% of P^{32} was excreted in the urine and 5% in the feces. It is possible that the small amount in the feces came from contamination with urine since an exact separation of these two products of excretion is difficult. These data demonstrate, however, that a large portion of the P^{32} has been excreted from the body 30 hours following a single injection of TEPA*.

Summary. The distribution of P^{32} following the injection of tagged TEPA* resulted in a fairly uniform distribution of P^{32} activity throughout the tissues of the body, but there was a greater concentration in the testes and a smaller concentration in bone as compared with injection of inorganic phosphate. There was a gradual accumulation of P^{32} in the spleen over a period of 30 hours following injection of TEPA*, a result not observed with inorganic phosphate. Approximately 60% of the P^{32} activity in serum or plasma was associated with the globulin fraction, the remainder being in solution after precipitation of all proteins. About 20% of P^{32} was associated with nuclei and/or relatively heavy cellular components from liver, testes, and tumor tissue, most of the remaining activity being found in the protein-free filtrate. Some 65% of the activity was excreted over a period of 30 hours.

We are indebted to Dr. C. M. Judson of the Stamford Laboratories, American Cyanamid Co., for assistance in the preparation of the tagged TEPA.*

---

1. Crossley, M. L., Allison, J. B., Wainio, W. W., and Muenzen, J. B., *J. Nat. Cancer Inst.*, 1951, v12, 305.

2. Crossley, M. L., Allison, J. B., and Muenzen, J. B., PROC. SOC. EXP. BIOL. AND MED., 1952, v80, 452.

3. Crossley, M. L., Allison, J. B., Parker, R. P., Kuh, E., and Seeger, D. R., in press.

4. Schneider, W. C., and Hogeboom, G. H., *Cancer Research*, 1951, v11, 1.

Received May 26, 1953. P.S.E.B.M., 1953, v83.

Valve-Like Action of Segment of Aorta Communicating Between Chambers of Left Atrium and Left Ventricle.* (20371)

RICHARD P. LASSER AND LEO LOEWE.

From Jacques Loewe Research Foundation Laboratories, Jewish Hospital of Brooklyn.

This is a report of the experimental testing of a concept which may prove to be useful in the surgical correction of mitral valve insufficiency. It was reasoned that a simple flexible tube leading from the atrium, through the mitral valve orifice and opening into the chamber of the left ventricle would itself function as a primitive valve. What was meant by a primitive valve was merely that the flow of fluid from the atrial end of the tube into ventricle during diastole would be unimpeded whereas retrograde flow through the communicating tube during systole would not take place. It was felt that retrograde flow would be prevented by the abrupt elevation of pressure within the ventricular cavity, which being exerted equally on all sides of the ventricular end of the tube, would simply collapse the lumen by apposing the walls. When the ventricular pressure declined at the termination of systole and finally fell below that existing within the atrial end of the tube, the walls would again be forced open and the lumen restored.

Methods. Adult dogs were anesthetized with Nembutal and the heart was exposed by removal of the 5th and 6th ribs on the left side. Respiration was maintained by a pneumatic balance respirator supplying intermittent positive pressure oxygen. In these acute experiments, the intracardiac tube was fashioned from sections of aorta. Both dog and cattle aortic segments have been used. The latter were employed because of their greater diameter. The insertion into the heart of these tubes was arranged so as to yield data concerning the validity of the anticipated valve-like action. Problems of the behavior of this system under long term conditions were not approached. A blunt probe bearing a heavy silk suture was inserted through the left ventricular wall at the apex and was passed through the mitral valve into the left atrial appendage and brought out through the atrial wall at this point. The suture was removed from the probe and joined to the lower end of the aortic segment. The wall of the atrial appendage was cut open around the initial probe opening and the aortic segment was drawn through this atrial opening into the ventricular cavity. The suture was then fixed to the ventricular myocardium to hold the ventricular end of the aortic segment in place and the atrial appendage was sutured to the adventitia of the proximal end of the aorta to effect a water-tight closure. Pressure within the aortic segment was determined through a No. 8 F cardiac catheter which was inserted into the portion of the aortic segment lying within the ventricular cavity and then slowly withdrawn into the atrial portion. The portion of the aorta which protruded through the atrial chamber and opened to the outside was kept sealed about the catheter during the determinations of pressure. The recordings of pressure were obtained with a Sanborn electromanometer and a direct writing 4 channel Polyvisocardiograph. The volume of flow of fluid through this intracardiac tube was determined by attaching its open end to wide lumen polythene tubing which led to a saline filled reservoir. The pressure at which flow took place was ascertained by measuring the height of the column of fluid at the start of a one-minute flow period and its height at the conclusion. The average of these 2 values was taken at the mean pressure for that particular flow period. Intraventricular injections of l-arterenol were given to observe the effect of great increases in ventricular pressure upon the pressure within the atrial end of the segment. In order to eliminate any possible valve-like action of the mitral valve or anulus fibrosis upon the aortic segment, flows were determined through a rigid tube passed into the aortic segment to a point just beyond the mitral valve. Animals were sacrificed at

---

* Aided by a grant from the Udo M. Reinach Fund.

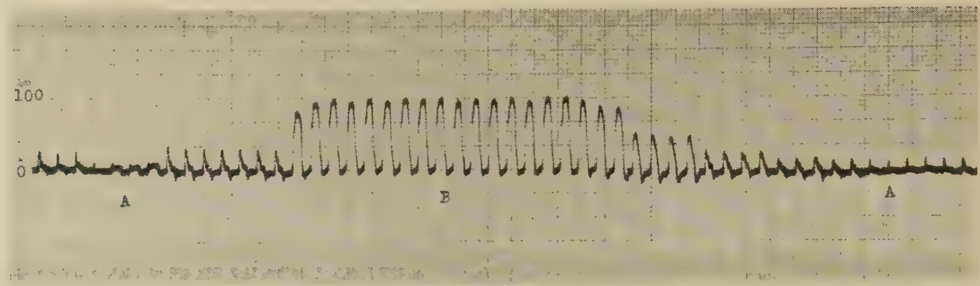


FIG. 1. Pressure pulses within the intracardiac aortic segment. These pressures were recorded as a cardiac catheter was slowly advanced from the atrial portion of the aortic segment (A), into the ventricular portion (B), and then withdrawn into the atrial portion (A). Pressure in mm of Hg is shown at the left. Speed of paper = 10 cm/sec.

the conclusion of the determinations and the proper position of the aortic segment was verified in all experiments.

Results. Pressure values were obtained in 9 dogs within the ventricular and atrial portions of the aortic segment. The mean value and standard deviation of the systolic pressures within the ventricular portion were 104 ± 12.5 mm Hg. Diastolic pressures in the same area ranged between zero and 4 mm Hg. The mean value and standard deviation of pressures within the atrial portion were 12 ± 3.4 mm Hg. The average pressure of 12 mm Hg within the atrial portion is not much greater than that observed normally in the left atrium of dogs (2-10 mm Hg) and would be compatible with a pulmonary capillary pressure well below that necessary to produce pulmonary edema.

Recordings of pressure were made in 3 animals during acute hypertension resulting from injection of l-arterenol. Ventricular systolic pressures were raised to heights of 360, 280 and 330 mm Hg. The corresponding pressures within the atrial portion of the aortic segment were, respectively, 16.0, 16.3, and 20.0 mm Hg. The existence of a great pressure differential between the ventricular and atrial portions of the aortic segment is offered as evidence as the predicted valve-like action. It might be pointed out that this change in pressure occurs abruptly. This is shown in Fig. 1 which is a typical tracing obtained by slowly advancing a cardiac catheter from the atrial to the ventricular portion of the aortic segment and then slowly withdrawing it.

TABLE I. Flow Values in 8 Dogs.

	Flow (cc saline), min.	Mean pressure, mm Hg
	250	10
	240	24
	185	17.6
	400	17.6
	197	15.8
	220	15.6
	225	15.1
	125	19.0
Avg	230	16.8

In order to demonstrate that ventricular filling would take place through this aortic segment, observations were made on the volume of saline which flowed from a reservoir through the aortic segment into the left ventricle. By raising or lowering the reservoir various levels of pressure could be maintained. The average flow per minute was 230 cc with a range of 125 to 400 cc and the average pressure at which these flows took place was 16.8 mm Hg with a range of 10 to 24 mm Hg (Table I). During the period of saline flow from reservoir to ventricle, no reverse flow of blood from ventricle to reservoir was observed. Ventricular systole merely caused an abrupt cessation of orthograde flow, at which time, a marked water-hammer effect was observable. If the protruding end of the aortic segment remained open to the atmosphere some back flow of blood was observed. This was the result of retrograde filling of the tube during diastole and the expulsion of this blood when ventricular systole reduced its volume. The maintenance of a modest continuous pressure from the saline reservoir completely prevented this back flow.

Summary. The valve-like action of a simple segment of aorta leading from the left atrium into the cavity of the left ventricle has been demonstrated. Orthograde flow from a reservoir of saline through the aortic tube into the left ventricle has been shown to occur in the living animal. No regurgitation has been observed. This observation has been documented by means of pressure determinations obtained within the portion of aorta lying in the left ventricle and that in the left atrium. An average systolic pressure of 104 mm Hg was observed in the ventricular portion and an average mean pressure of 12 mm Hg in the atrial portion. This demonstration of orthograde flow during diastole without retrograde flow during ventricular systole is felt to constitute evidence of an effective valve-like action.

The authors wish to express their gratitude to Mr. Paul Geller, Dr. Ruth Kaslow, and Mrs. Selma Rachlin for their assistance.

Received April 16, 1953. P.S.E.B.M., 1953, v83.

Influence of Low Protein Diet on Distribution of D-Amino Acid Oxidases in Rats.* (20372)

C. HOCH-LIGETI. (Introduced by C. L. Gemmill.)
From the Department of Pathology, University of Virginia Medical School, Charlottesville, Va.

In connection with a series of experiments on the influence of nutrition on the development of hepatic tumors, the effect of nutritional factors on the amino acid oxidases was studied. A decrease of amino acid oxidases was found previously in hepatic tumors(1-3) as well as with the livers of rats bearing transplanted liver tumors.

The present investigation attempted to answer the following questions: a) do nutritional factors influence the levels of amino acid oxidases, b) is such an influence general or restricted to any one organ, and c) does the administration of an estrogen together with a low protein diet produce any changes in the level of amino acid oxidase. The impairment of the detoxication of estrogens on low protein diet has been described(4). Experiments on the possibility of the production of hepatic tumors by estrogens added to a low protein diet are being carried out in this laboratory. In the rat, amino acid oxidases are present in appreciable quantities only in the liver and in the kidneys(5). In both organs a general D-amino acid oxidase has been demonstrated. In addition, a specific D-aspartic acid oxidase has been found in pig kidneys(6). In the present experiment the amino acid oxidases were studied both in the liver and in the kidney. Although next to D-L-alanine, D-aspartic acid was used as substrate, no attempt was made to separate the activity of the general D-amino acid oxidase from the specific D-aspartic acid oxidase.

Materials and methods. Young Wistar rats of both sexes, weighing 70-90 g at the beginning of the experiment were divided into 3 groups; one was kept on a full diet, one on a low protein diet, and a third group on a low protein diet with the addition of alpha estradiol. Rats fed on rat biscuits served as control on full diet. The low protein diet consisted of: Protein (vitamin-free casein) 8.5%, carbohydrate (sugar and cornstarch) 70%, fat (lard and vegetable oil) 17%, salt mixture 3.5%, and cod liver oil 1%. Vit. B and E were given to 1000 g of diet in the following amounts: thiamin hydrochloride 6 mg, pyridoxin 20 mg, inositol 15 mg, choline dihydrogenphosphate 100 mg, calcium pantothenate 20 mg, and alpha tocopherol 30 mg. Food and water were given *ad lib.* Alpha estradiol was fed to half the number of the rats on the experimental diet. Each of these rats received orally by eye dropper 5 μg estradiol dissolved in 0.1 ml olive oil daily with the exception of Sundays. Rats were killed at intervals, after having been on the experimental diet, with and without addition of estradiol for at least 3 months. The D-amino-acid oxidase was determined by measuring the oxygen uptake of liver and kidney homogenates in the presence of substrate by the Warburg technic. Each vessel contained 200 mg of homogenate, 0.3 ml of a 0.3% D-L-alanine or D-aspartic acid and M/15 phosphate buffer of pH 8.4. The central cup contained 0.2 ml of 20% potassium hydroxide. The total fluid volume was 3 ml. The experiments were carried out in duplicate at 38°C. Readings were taken every 10 minutes through 1-3 hours. The activity of D-amino-acid oxidase was calculated as milliliters of oxygen taken up by one g of wet tissue during one hour.

---

* This investigation was supported by a research grant from the National Cancer Institute, National Institutes of Health, Public Health Service.

Results and discussion. All rats on low protein diet showed fatty livers; the male rats receiving estradiol showed pronounced cirrhosis and 4 out of 28 had cholangiomas. (This part of the experiment is now being repeated and will be reported at a later date.) The weights of the livers of rats on low protein diet were increased compared with the liver weights of rats on the normal full control diet. The other organs, with the exception of the genital organs of rats receiving estradiol, did not show significant differences between the rats on the experimental and on the control diet.

Since the effect of feeding the low protein diet on the D-amino acid oxidase was the same whether the rat received this food for 3 or for 9 months, and since it was found to be the same in the organs of male and female rats the results are discussed together. In every experiment the oxygen uptake of liver and of kidney was the same with alanine or with aspartic acid as substrate. Table I shows that the level of D-amino acid oxidases of the kidneys are the same in the control rats on full diet and in both groups of experimental animals on the low protein diet. The level of D-amino acid oxidases in the livers of rats on low protein diet dropped to about half of the values found in the rats receiving a normal full diet. No difference was found in the activity of D-amino acid oxidase in the liver of the rats on low protein diet on addition of

TABLE I. D-Amino Acid Oxidases in Livers and Kidneys of Rats on Low Protein Diet.

						Liver oxidase, ml O_2/g tissue/hr						Kidney oxidase, ml O_2/g tissue/hr			
		Body wt		Liver wt, % of body wt		D-L-alanine		D-aspartic acid		Kidney wt, % of body wt		D-L-alanine		D-aspartic acid	
Treatment	No. of rats	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean
Control, full diet	10	123 217	174	3.4 5.1	4.10	365 610	449	325 535	434	.70 1.18	.86	240 480	342	220 475	338
Low protein diet	8	140 250	172	3.4 7.9	5.77	159 240	211	139 274	211	.64 1.17	.83	197 540	300	170 590	301
Low protein diet with estradiol	13	107 212	163	3.7 7.2	5.00	148 330	248	126 283	244	.51 1.38	.70	190 480	318	190 460	292

estradiol, though cholangio-fibrosis, and cirrhosis of the liver was more marked in the latter groups. The increase in the weight in the liver of rats on low protein diet is proportionally much smaller than the decrease in the level of the amino acid oxidases, accordingly a possible dilution of the enzyme in the liver cannot be taken as an explanation. In previous experiments(7) both components of the D-amino acid oxidase, the co-ferment riboflavin and the protein was estimated separately in liver of rats on protein free diet. The decrease in the amino acid oxidase in the livers could not be accounted for by the decrease of the riboflavin in the livers, and was thought to be due to a decrease of the specific enzyme protein. The amino acid oxidases in other organs were not studied. In view of the active participation of the liver in the protein metabolism, the finding in the present experiment that on reduction of dietary proteins a decrease of the amino acid oxidase occurred in livers only, might be of physiological importance.

Summary. 1. D-amino acid oxidases in livers and kidneys of rats on a low protein diet with or without addition of alpha estradiol have been compared with those of rats on a full diet. 2. The levels of D-amino acid oxidases in the kidneys was the same in all 3 groups. 3. In livers of rats on low protein diet, irrespective of feeding of alpha estradiol, the level of D-amino acid oxidases dropped to about half of that found in rats on full diet.

The author wishes to thank the Schering Corp., Bloomfield, N. J., for donating the alpha estradiol used in this experiment.

---

1. Westphal, U., *Z. Physiol. Chem.*, 1942, v276, 191; 1944, v281, 89, 94.

2. Shack, J., *J. Nat. Cancer Inst.*, 1942-43, v3, 389.

3. Lan, T. H., *Cancer Research*, 1944, v4, 43.

4. Shipley, R. A., Gyorgy, P., Proc. Soc. Exp. Biol. and Med., 1944, v57, 52.

5. Birkhofer, L., Wetzel, R., *Z. Physiol. Chem.*, 1940, v264, 31.

6. Still, J. L., Buell, M. V., Knox, V. E., Green, D. E., *J. Biol. Chem.*, 1949, v179, 831.

7. Seifter, S., Harkness, D., Rubin, L., Muntwyler, E., *J. Biol. Chem.*, 1948, v176, 1371.

Received May 26, 1953. P.S.E.B.M., 1953, v83.

Histochemical Studies on Glycogen Deposition in Uterus of the Rat*
III. Effect of Starvation. (20373)

Walter J. Bo† and William B. Atkinson.
From the Department of Anatomy, University of Cincinnati College of Medicine, Cincinnati, O.

It has been shown recently that glycogen is deposited in the smooth muscle of the uterus of the rat under the influence of estrogen, whereas progesterone, androgen and adrenal cortical extract each fail to produce this effect (1-3). This action of estrogen on the uterus is independent of both the adrenal cortex and hypophysis(2,3), but is, in a sense, analogous to the role played by the hormones of the latter organs in the deposition of glycogen in the liver and skeletal muscle. The glycogen content of the liver and skeletal muscle remains within normal limits in adrenalectomized(4) and hypophysectomized(5) animals provided a good nutritional state is maintained. Relatively short periods of fasting, however, result in a sharp reduction of liver glycogen in adrenalectomized animals and of both liver and skeletal muscle glycogen in hypophysectomized animals, although normal levels are promptly restored following treatment with the appropriate adrenal cortical or hypophyseal hormones(6,7).

The glycogen content of the uterus of intact and estrogen-treated castrate rats is not reduced by fasting(3). Whether estrogen can induce glycogenesis in the uterus after depletion of carbohydrate reserves, however, is not

---

* This investigation has been aided by a grant from the Montgomery County Society for Cancer Control, Dayton, O.

† Montgomery County Cancer Fellow. Present address: Department of Anatomy, Univ. of North Dakota Coll. of Med., Grand Forks, N. D.

TABLE I. Amount and Distribution of Glycogen in Uteri and Livers of Fasted and Unfasted Rats Treated with Estrogen.

		Uterus					
		Myometrium		Endometrium			
Treatment*	No. of animals	Outer long.	Inner circ.	Epithelium	Glands	Stroma	Liver
Not fasted							
Ovariectomized	5	++++	++	+	0	0	++++
Ovariectomized-hypophysectomized	6	++++	++	++	0	0	+++
Fasted							
Ovariectomized	5	++++	++	+	0	0	+
Ovariectomized-hypophysectomized	7	++++	++	+	0	0	0

* All animals given one subcut. inj. of 20 μg of estradiol dipropionate and sacrificed 48 hr later.

known. The present experiments were designed, therefore, to determine the efficacy of estrogen in inducing glycogen deposition in the uteri of ovariectomized and ovariectomized-hypophysectomized rats starved prior to the beginning of hormonal treatment.

Materials and methods. Twenty-three adult female Wistar rats were bilaterally ovariectomized and divided into 4 groups as follows: 1) 5 animals were injected with 20 μg of estradiol dipropionate[‡] at 3 P.M. of the 10th day after ovariectomy and were sacrificed 48 hours later; 2) 5 animals were treated as in Group 1 except all food was withheld beginning at 9 A.M. of the day on which they were injected with estrogen; 3) 6 animals were hypophysectomized on the 5th day after ovariectomy, injected with 20 μg of estradiol dipropionate at 3 P.M. on the 10th day after hypophysectomy and were sacrificed 48 hours later; 4) 7 animals were treated as in Group 3 except all food was withheld after 9 A.M. of the day on which they were injected with estrogen. When not fasted, the rats were maintained on Purina Chow *ad libitum*. Drinking water was available at all times. Crystalline hormone was injected subcutaneously, the dose being dissolved in 0.20 ml of cottonseed oil. At the termination of the experiment the animals were killed with chloroform and were autopsied immediately. They were examined grossly to ascertain the complete removal of the glands in question. Small pieces of uterus and liver were fixed in cold picro-alcohol-formalin and glycogen was subsequently demonstrated by the periodic acid-leucofuchsin technic previously described(1).

Observations. The results are summarized in Table I. In both the ovariectomized and ovariectomized-hypophysectomized rats fed *ad libitum* the deposition of glycogen in the uterus was similar to that described in our previous studies, *i.e.*, considerable amounts accumulated in the longitudinal layer of the myometrium and in the mesometrial region of the circular layer, smaller amounts being found in the remainder of the circular muscle. No glycogen was detected in either the epithelium of the endometrial glands or in the cells of the stroma.

In our previous studies glycogen deposition was not observed in the lining epithelium of the uterine lumen. However, in the present experiments, due presumably to the considerably larger dose of estrogen used, glycogen appeared in the lumenal epithelium. It was somewhat variable in amount and distribution in each uterus, some areas of the epithelium being free of the material, others containing small to moderate quantities. The over-all deposition was somewhat greater in the hypophysectomized castrates than in the castrates with intact hypophyses, but probably no special significance should be ascribed to this observation.

The livers of the fed ovariectomized rats showed extensive glycogen deposition, the parenchymal cells throughout the hepatic lobules being more or less uniformly filled with granules. In the fed hypophysectomized castrates, however, the amount of gylcogen in the cells in the outer one-third of the lobules was considerably reduced.

[‡] Estradiol dipropionate used in these studies supplied through the courtesy of the Ciba Pharmaceutical Products, Inc., Summit, N. J.

The glycogen content of the uteri of the starved ovariectomized and ovariectomized-hypophysectomized rats was not discernibly different from that of the fed ovariectomized animals described above. The liver glycogen in the starved animals, on the other hand, was greatly reduced compared with that present in the fed rats. In the starved ovariectomized animals there was sparse deposition only in the cells in the central portions of the lobules, whereas in the hypophysectomized castrates glycogen was completely absent.

Discussion. Carbohydrate metabolism is regulated to a considerable extent by the pancreas, anterior pituitary and adrenal cortex through their hormonal influence on the utilization of glucose by skeletal muscle and other peripheral tissues and through their effects on the storage and release of glucose by the liver. It should be noted that these various endocrine factors which affect carbohydrate metabolism in the living animal are not necessarily essential to the biochemical processes concerned in the storage and utilization of sugars, *e.g.*, in the hypophysectomized-adrenalectomized animal the levels of blood glucose and liver glycogen are maintained within normal ranges provided the animals are well nourished. Furthermore, many of the biochemical processes involved in carbohydrate metabolism have been demonstrated to proceed *in vitro* in the absence of all hormonal influence.

Little interest has been taken to date in the carbohydrate metabolism of smooth muscle or in its regulation by the endocrine glands. The observations of Walaas(3) and of the present authors(1,2) on the glycogenetic effect of estrogen on the uterine muscle in the rat have indicated that the synthesis and deposition of glycogen in this tissue proceed in the absence of the pituitary and adrenal cortex; the present study has demonstrated that estrogen remains effective as a stimulant for glycogenesis even if the carbohydrate stores of the animal are first depleted by starvation. The biochemical mechanisms involved remain obscure. It seems reasonable to assume, however, that the effect of estrogen is directly on the uterine muscle either through facilitation of glycogenesis from blood glucose, despite the lowered concentration of the latter, or through promotion of glyconeogenesis.

The deposition of glycogen in the uterine epithelium of the animals in the present study runs counter to our earlier experience in the intact cyclic rat and in the castrate treated with smaller amounts of estrogen. The present dosage of 20 μg of estradiol dipropionate is double or more that previously used and was selected in hope that a larger amount of glycogen would be deposited in the uterine muscle, thus facilitating microscopic study. Since we have re-examined our earlier material and have found no more than traces of glycogen deposited in the uterine epithelium of the intact cyclic rats, the present epithelial response may be considered to be "nonphysiologic".

Summary. Relatively short periods of fasting result in the rapid disappearance of glycogen from the liver and skeletal muscle of hypophysectomized animals. The present experiments have demonstrated that, in spite of the depletion of carbohydrate reserves in the starved hypophysectomized castrate rat, estrogen promotes the deposition of glycogen in the uterine musculature in amounts comparable to those deposited in well-nourished animals. In this respect this action of estrogen on the uterus is analogous to that of certain of the hormones of the anterior hypophysis and adrenal cortex on the deposition of glycogen in the liver and skeletal muscle.

---

1. Bo, W. J., and Atkinson, W. B., *Anat. Rec.*, 1952, v113, 91.
2. Atkinson, W. B., and Crafts, R. C., *Anat. Rec.*, 1952, v113, 101.
3. Walaas, O., *Acta. Endocrinol.*, 1952, v10, 175.
4. Kuriyama, S., *J. Biol. Chem.*, 1918, v34, 287.
5. Russell, J. A., PROC. SOC. EXP. BIOL. AND MED., 1936, v34, 279.
6. Long, C. N. H., Katzin, B., and Fry, E. G., *Endocrinol.*, 1940, v26, 309.
7. Bennett, L. L., and Perkins, R. Z., *Endocrinol.*, 1945, v36, 24.

Received May 27, 1953. P.S.E.B.M., 1953, v83.

Effect of Benzimidazole on Experimental Poliomyelitis in Mice and Monkeys.* (20374)

Gordon C. Brown, Donald E. Craig, and Alexander Kandel.

From the Department of Epidemiology, School of Public Health, University of Michigan, Ann Arbor.

As components of nucleo proteins, the purines and pyrimidines are intimately involved in biological systems. Their analogues, which interfere with the synthesis of nucleic acids, are known to affect the metabolic activity of certain bacteria(1-6) and viruses (7-10). One of these, benzimidazole, in which the pyrimidine ring has been replaced by benzene, was found to be active in tissue cultures against the viruses of vaccinia(11), tobacco mosaic(12), and psittacosis(13). More recently, the author(14) has demonstrated that this compound, as well as other inhibitors of nucleic acid synthesis, effectively suppresses the growth of poliomyelitis virus in tissue culture. The present report describes the effect of benzimidazole on poliomyelitic infection in animals.

Materials and methods. Swiss Albino mice weighing 17 to 20 g were individually weighed to insure exactitude in the drug administration. Toxicity tests revealed that mice of this weight could tolerate without ill effect 250 mg of commercial benzimidazole† per kilo of body weight when administered subcutaneously for as long as 21 days. The type 2 rodent adapted viruses (Lansing and MEF-1) used in tests with mice had been prepared in large quantities and stored as pools at —70°C in sealed ampoules until used. Mice were infected by intracerebral injections of 0.03 ml of the appropriate dilutions of virus and were observed daily for at least 21 days for signs of paralysis. The virus used in tests with monkeys was a pool of Mahoney strain, type 1, poliomyelitis virus from infected monkey central nervous tissue which was infective either intracerebrally, subcutaneously, or intraperitoneally in dilutions of at least 10^{-3}. The monkeys were observed for 21 days, and if not previously paralyzed, were posted at that time and sections of the nervous system were examined for histological evidence of poliomyelitis.

Experimental tests in mice. Lansing strain. Six separate tests were performed in mice with the Lansing strain of Type 2 poliomyelitis virus which had a PD_{50} titer of 10^{-4} or higher. Four dilutions of virus, 10^{-1} through 10^{-4}, were employed in groups of either 20 or 50 mice each for both the drug treated and the virus control mice. The animals received 250 mg/kg benzimidazole either once on the day of virus inoculation (acute administration) or daily for the duration of the experiment (chronic). No difference in morbidity or mortality was observed between those mice receiving drug on the acute schedule or those on chronic administration. Consequently, the results of all tests are considered irrespective of the schedule of drug administration. Table I shows the cumulative results of 6 separate experiments in mice employing 4 dilutions of the Lansing strain of poliomyelitis virus and 250 mg of benzimidazole per kilo of body weight. The only dilution of virus which killed more control than benzimidazole treated mice was 10^{-4}. The probability of chance occurrence of this difference, however, is $P = .01$, and therefore the 13% decrease in mortality seen in the drug treated groups represents statistical significance. It will be seen that the arithmetic mean of the incubation period in mice receiving drug increased 3 or 4 days with each 10-fold dilution of virus. A corresponding but not as marked an increase was seen in the control mice. However, the incubation period was almost as great in the 10^{-4} controls as in the drug treated mice suggesting that the major effect of benzimidazole was on the incidence of infection rather than in prolonging the incubation period. The cumulative mortalities of both control and drug treated mice receiving the 10^{-4} dilution of Lansing virus in 6 experiments are presented graphically in Fig. 1.

---

* Aided by a grant from the National Foundation for Infantile Paralysis.

(Presented at Annual Meeting of Am. Assn. of Immunologists, Chicago, Apr. 6, 1953.)

† Nutritional Biochemicals Corp., Cleveland, O.

TABLE I. Cumulative Results of 6 Experiments in Mice with Subcutaneously Administered Benzimidazole, 250 mg/kg and Lansing Strain Poliomyelitis Virus.

	Dilutions of virus							
	10^{-1}		10^{-2}		10^{-3}		10^{-4}	
	Drug (149)*	Con. (165)	Drug (229)	Con. (169)	Drug (225)	Con. (169)	Drug (232)	Con. (172)
A.M.†	4.6	5.3	7.57	5.93	10.75	9.5	14.5	13.0
Range	2–15	2–20	2–19	2–20	2–28	2–29	5–28	2–28
% mort.	99	98	98	98	86	82	46	59
Range mort., %	96–100	95–100	94–100	94–100	70–100	57–100	36–60	45–85

* Total No. of mice in all tests.

† Arithmetic mean of incubation period in days.

MEF-1 strain. Since the only dilution of the Lansing strain at which benzimidazole appeared to have an effect in mice was 10^{-4}, the approximate PD_{50} of that virus, similar tests at or near the end point of virus titer were performed using the MEF-1 strain of Type 2 poliomyelitis virus which usually infected mice when diluted as much as 10^{-5} and occasionally in a 10^{-6} dilution. The same concentration of benzimidazole (250 mg/kg) was inoculated and except for greater numbers of mice per dilution the technics were identical to those used in the above-described tests. Benzimidazole, however, was not effective in decreasing the mortality caused by this strain of poliomyelitis virus. In fact, more of the treated mice than the controls developed the disease with all 3 dilutions of this agent. Since the intracerebral inoculation of virus represents an extremely severe challenge to the efficacy of any drug which might influence infection, suckling mice between the ages of one and 3 days were inoculated intraperitoneally with 0.1 ml of a 10% suspension of the MEF-1 strain of virus. Some of these were treated subcutaneously with 250 mg/kg benzimidazole and some with 100 mg/kg. Thirty-three of the animals received the virus alone and 16 developed the disease. Of the mice receiving 100 mg/kg benzimidazole 23 of 30 became paralyzed as did all 6 who received 250 mg/kg. This indicates that far from preventing infection in suckling mice inoculated with virus intraperitoneally, the drug apparently enhanced their susceptibility to poliomyelitis.

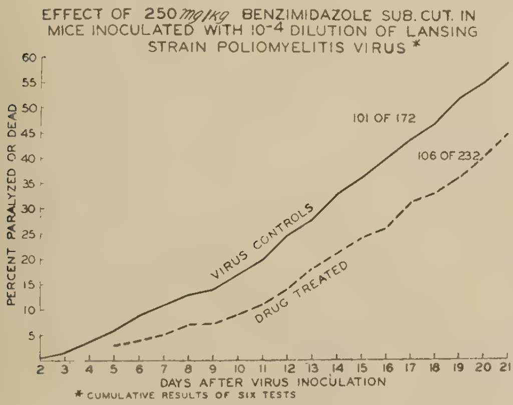


FIG. 1.

Tests in monkeys. The effect of benzimidazole on infection in monkeys was investigated. Ten rhesus monkeys (*Macaca mulatta*) were inoculated intracerebrally with 0.5 ml of a 10^{-2} dilution of the Mahoney strain of Type 1 virus. One-half of the animals received 250 mg of benzimidazole per kilo of body weight subcutaneously for 3 days beginning on the day of virus inoculation. In another test 20 cynomolgus monkeys (*Macaca iris*) were inoculated intraperitoneally with 1.0 ml of a 10^{-2} dilution of the same pool of virus and half of these were treated for 3 days with 200 mg of benzimidazole per kilo. The results of these tests are shown in Table II. All the drug treated animals as well as the controls developed paralysis regardless of the route of inoculation. The only difference between the 2 groups of monkeys was evidenced by a slightly longer and more irregular incubation period in the drug treated groups. This slight difference, however, was emphasized by the regularity with which the virus controls developed the paralytic disease, most of them within 24 hours of each other. In a third test 10 cynomolgus monkeys were treated with 250 mg of the drug per kilo daily for 5 days prior to and for 2 days after the subcutaneous

TABLE II. Effect of Benzimidazole on Infection with Mahoney Strain of Poliomyelitis Virus in Monkeys.

Monkey	Virus: Route	s.c. drug, mg/kg	Day of paralysis	
			Control	Benzimidazole
Rhesus (10)	10^{-2} i.c.	250 3 × $\bar{p}$	7, 7, 8, 8, 8 Avg = 7.6	7, 8, 9, 10, 10 Avg = 8.8
Cyno. (20)	10^{-2} i.p.	200 3 × $\bar{p}$	6, 7, 7, 7, 7, 7, 7, 7, 8, 11 Avg = 7.4	7, 7, 8, 8, 8, 8, 9, 10, 11, 12 Avg = 8.8
Cyno. (20)	10^{-3} s.c.	250 5 × $\bar{a}$ 2 × $\bar{p}$	9, 12, 12, 12, 14, 14, 16, 17, —, — Avg = 13.2	8, 9, 12, 13, 14, 14, 21, —, —, X Avg = 13.0

inoculation of 1.0 ml of a 10^{-3} dilution of Mahoney virus. Seven of these animals developed paralysis as compared with 8 of the 10 controls, and the average incubation periods of the 2 groups were almost identical. It was apparent that benzimidazole, under the conditions tested, had little or no effect upon the course of poliomyelitic infection in monkeys.

Discussion. Few compounds which show antiviral activity in tissue culture are similarly effective in animals. This is probably due to the inability to exhaust or replace completely a given metabolite from the tissues of the intact host. Furthermore, the quantity necessary to accomplish this state of deprivation is usually far in excess of the toxic levels which are tolerated by the animal. The results of experiments with benzimidazole, therefore, are singular in this regard. Although the decrease in mortality is moderate and is observed only with minimal infective quantities of Lansing strain poliomyelitis virus, the influence of this compound on the process of disease is evident in the rather consistently prolonged incubation period even with larger quantities of virus. The drug itself is well tolerated by the animal without ill effect for the duration of these experiments. It is possible, therefore, that this represents an example of reducing the availability of a metabolite essential for the growth of virus without adversely affecting the host itself.

It would be far too optimistic to expect to find a drug or chemical which would completely prevent the occurrence of poliomyelitis in experimentally infected animals. However, when many compounds are tested without observing the slightest effect on either the incubation period or the mortality, as is the case in this laboratory, the results obtained in the present study appear highly suggestive that the investigation of this general class of compounds offers a reasonable approach to the chemotherapy of this disease.

Summary. 1. The influence of benzimidazole on experimental poliomyelitis in mice and monkeys was investigated. The subcutaneous administration of 250 mg/kilo prolonged the incubation period of infection with the Lansing strain of Type 2 poliomyelitis in mice and reduced the mortality caused by minimal quantities of this virus. 2. Infection of adult mice with intracerebrally inoculated MEF-1 strain of Type 2 virus was not affected by this compound which even appeared to enhance infection in suckling mice inoculated intraperitoneally with this same strain. 3. In monkeys the incubation period of infection with the Mahoney strain of Type 1 virus was prolonged slightly by limited treatment with benzimidazole, but the mortality was not reduced. More intensive treatment was without effect. 4. The advisability of further work with this and structurally similar compounds is discussed.

---

1. Woolley, D. W., *J. Biol. Chem.*, 1944, v152, 225.

2. Klotz, I. M., and Mellody, M., *J. Bact.*, 1948, v56, 253.

3. Edwards, P. C., Starling, D., Mattocks, A. M., and Skipper, H. E., *Science*, 1948, v107, 119.

4. Hitchings, G. H., Falco, E. A., and Sherwood, M. B., *Science*, 1945, v102, 251.

5. Elion, G. B., Vander Werff, H., and Falco, E. A.,

J. Biol. Chem., 1948, v174, 765.

6. Strandskov, F., and Wyss, O., *J. Bact.*, 1945, v50, 237.
7. Thompson, R. L., Wilkin, M. L., Hitchings, G. H., Elion, G. B., Falco, E. A., and Russell, P. B., *Science*, 1949, v110, 454.
8. Friend, C., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 150.
9. Moore, A. E., and Friend, C., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 153.
10. Commoner, B., and Mercer, F. L., *Arch. Biochem. and Biophys.*, 1952, v35, 278.
11. Thompson, R. L., *J. Immun.*, 1947, v55, 345.
12. Commoner, B., Personal communication.
13. Morgan, H. R., *J. Exp. Med.*, 1952, v95, 277.
14. Brown, G. C., *J. Immun.*, 1952, v69, 441.

Received May 4, 1953. P.S.E.B.M., 1953, v83.

Protection Against Radiation Lethality: Effect of β-Mercaptoethylamine.* (20375)

R. RUGH† AND S. C. WANG.‡

From the Radiological Research Laboratory and Department of Physiology, College of Physicians and Surgeons, Columbia University.

The search for some agent which will protect man and animals against, or alleviate them from, the effects of accidental or overexposure of ionizing radiations has been the incentive for many investigations. The most promising of the many chemical compounds used has been *l*-cysteine hydrochloride. Patt and his coworkers(1) found that if mice were injected with cysteine before exposure to x-irradiations, their tolerance would be greatly increased and many would survive doses which would normally prove fatal to 100% of the untreated controls. This led others to investigate the amines, particularly those containing the —SH radical because of the suggestion that an extra supply of this radical might temporarily neutralize certain enzymes with respect to their normally high radiosensitivity(2). Recently, Bacq and his coworkers in Belgium investigated many such compounds. One of these was related to Patt's cysteine, namely cysteinamine or β-mercaptoethylamine. By injecting this compound into C_{57} black mice, they raised the minimum 100% lethal dose of x-rays from 700 r to 1300 r(3). They also found that this compound gave complete protection to mice exposed to 100% lethal dose of x-rays. In this paper the term "cysteinamine" is used instead of β-mercaptoethylamine in order to emphasize its similarity to cysteine which has been so effective in radiation protection studies.

The reasons for the present further investigation of cysteinamine as a protective agent against ionizing radiations are: (a) Variations in sensitivity make it impossible to interpolate results with C_{57} mice for the mice more commonly used in this country, namely the Swiss albino and CF_1 strains, and (b) Radiation studies using male mice exclusively are more reliable than those using females, or both sexes.

Materials and method. The Swiss albino mice were raised in our laboratories and were 6 months of age. The CF_1 were 2 months of age when purchased from the Carworth Farms. They were brought to a uniform weight of approximately 25 g at the time of irradiation. Some 659 mice were used in all. The cysteinamine was acquired in ampoules, labelled 1573 L and produced by the Labaz Laboratory in Belgium. Each ampoule consisted of 10 cc of a 10% solution. This was refrigerated until opened, and was used within a few minutes after dilution with physiological saline. The dilution was such that each cubic centimeter contained 3 mg of cysteinamine. Several parallel experiments were run with

---

* Made available through the courtesy of Prof. Z. M. Bacq of Liege, Belgium.

† Based upon work performed under Contract for the Atomic Energy Commission.

‡ Supported by a Grant from the National Institute of Neurological Disease and Blindness.

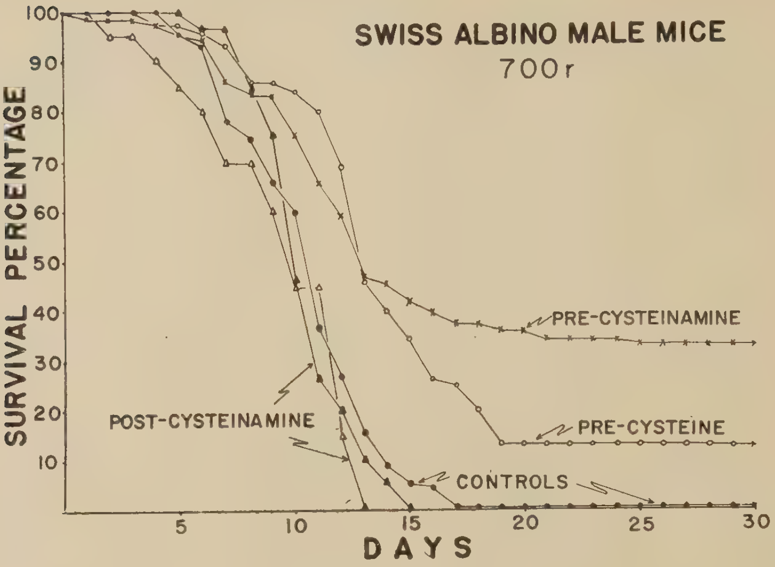


FIG. 1.

l-cysteine. This was made up so that each cubic centimeter contained 12.5 mg, and was neutralized to pH 7.25 with NaOH. The treatment consisted of intraperitoneal injections of 1 cc of either cysteinamine or cysteine. In most cases the injection was made within 5 minutes before x-irradiation, but, as noted below, in a few experiments single or multiple injections were made after irradiation. The mice were x-irradiated 5 at a time in a perforated and circular plastic cage measuring 14 cm in inside diameter and 4 cm in inside height. The calibration of the x-irradiation was made with the ionization chamber in the center of the closed cage. The distance from the target to the center of the mouse's body was 30 cm. Constant movement of the mice within the cage tended to balance out any very small difference that might exist between the center and the periphery of the cage. The physical constants of x-irradiation were: Westinghouse Quadrocondex constant-potential x-ray machine run at 2.0 KV, 15 MA, filtration 0.25 Cu and 0.50 Al, and a dose rate of 274 r/minute in air. The exposure was for 2′ 42″ for each group of mice. After x-irradiation the mice were returned to the animal room where they received the usual care, with food and water *ad libitum*. A daily record of deaths was taken for the experimental period of 30 days.

Experimental data. The injected dose of cysteinamine or cysteine was not varied. However, in some experiments the mice were injected prior to x-irradiation (pre-cysteinamine series) and in some they were injected just after x-irradiation (post-cysteinamine series). The data are presented in Fig. 1 and 2, which include all groups of animals, control as well as treated, with the survival values expressed as percentages.

Discussion. According to Bacq and Herve (3,5), and Bacq, Fischer, and Pirotte(6), cysteinamine is very quickly metabolized, disappearing from rabbit plasma within 15 minutes after injection and appearing in the urine within half an hour. It was estimated that about 1/3 of it is eliminated during the first half hour following injection, and mice x-irradiated one hour following injection received no protection thereby. This substance is relatively non-toxic to mice. The median lethal dose for C_{57} black mice has been determined as 0.35 mg/g of body weight and the minimum lethal dose for all animals studied was 0.5 mg/g of body weight(4). Mice receiving the dose of 3 mg I.P., used in these experiments, showed no ill effects whatsoever.

Bacq and his coworkers have found that the dose of x-irradiation required to kill all

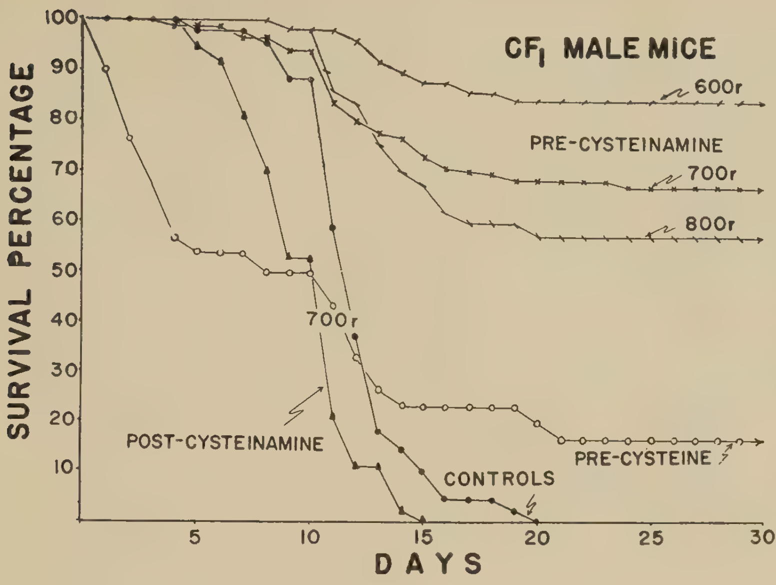


FIG. 2.

of the control C_{57} black mice within 8 days was 700 r whole body exposure, but that mice receiving prior injection of 3 mg of cysteinamine could tolerate as much as 1300 r or as well as did the controls at 700 r. Further, those receiving the cysteinamine and 700 r showed complete survival to the termination of the experiment in 24 days. The most plausible theory with regard to the mechanism of protection relates to the possibility that some —SH compounds partially or completely inhibit the deleterious action of free radicals, normally found in x-irradiated tissue, or they may even modify their nature in such a manner as to render them less harmful (see discussions of the possible protective mechanism of these compounds) (4,7-12). It is also thought possible that the effective —SH carrying compounds may combine temporarily with some radiosensitive enzymes, making them radioresistant(4). Cysteinamine is reported to be a powerful anti-mitotic agent in concentrations above 10^{-4} in tissue cultures(3,5). In cats and dogs it is reported to produce no change in arterial pressure and respiration (3,5). In rabbits it does show anti-inflammatory properties(13). Some physiological and cancericidal studies have been made in humans(14).

The experiments reported here indicate that cysteinamine is very effective in protection against the radiation syndrome when injected prior to exposure. Although the percentage of survival was not as striking as that reported by Bacq and his coworkers, we have observed that some 32% of the Swiss albino and some 67% of the CF_1 male mice exposed to the lethal dose of 700 r survived beyond the experimental period of 30 days. In fact, 97% of these have survived 45 days. Cysteinamine, if given after x-irradiation not only is ineffectual, but also increases the mortality rate, *i.e.*, the mice thus treated died more quickly as compared with the controls.

Over 100 male CF_1 survivors of 700 r x-rays, pre-treated with cysteinamine, are currently being tested for fertility. A goodly number have already proven to be fertile in spite of the high level of exposure. A complete blood analysis is being made and histopathological changes are being investigated. The results of these studies, to be reported in full at a later date, should cast more light on the mechanism of protection by cysteinamine. With the increasing use of ionizing radiations, both in therapy and diagnosis, the imperative aspect of standardizing the protective procedures is obvious.

Summary and conclusions. Some 32% of the Swiss albino and 67% of the CF_1 male mice injected intraperitoneally with β-mercaptoethylamine (*i.e.*, cysteinamine) prior to a lethal exposure of 700 r whole body x-irradiation survived the 30-day observation period. Some 97% of these are still alive 60 days after irradiation. The CF_1 mice, exposed to the higher dose of 800 r after injection, showed 58% survival while the controls, receiving only 700 r, all died within 20 days. Thus prior injection of this compound definitely affords radiation protection to mice. Injection after x-irradiation, whether single or multiple, had an adverse effect on survival.

1. Patt, H. M., Tyree, E. B., Straube, R. L., and Smith, D. E., *Science*, 1949, v110, 213.
2. Rugh, R., *Mil. Surgeon*, 1953, v112, 395.
3. Bacq, Z. M. et Herve, A., *J. Suisse Med.*, 1952, v40, 1018.
4. Herve, A., et Bacq, Z. M., *J. belge radiol.*, 1952, v35, 524.
5. Bacq, Z. M., et Herve, A., *Bull. Acad. Royale Med.*, 1952, v17, 13.
6. Bacq, Z. M., Fischer, P., et Pirotte, M., *Arch. internat. physiol.*, 1952, v60, 535.
7. Barron, E. S. G., and Dickman, S., *J. Gen. Physiol.*, 1949, v32, 595.
8. Limperos, G., *J. Franklin Inst.*, 1950, v249, 513.
9. Chapman, W. H., Sipe, C. R., Eltzholtz, D. C., Cronkite, E. P., and Chambers, F. M., *Radiol.*, 1950, v55, 865.
10. Graham, J. B., and Graham, R. M., *Cancer*, 1950, v3, 709.
11. Cronkite, E. P., Brecher, G., and Chapman, W. H., *Mil. Surgeon*, 1951, v109, 294.
12. Jacobson, L. O., *Cancer Research*, 1952, v12, 315.
13. Lecomte, T., et Borenstayn, C., *Compt. R. Soc. Biol.*, 1953, in press.
14. Bacq, Z. M., Bernard, J., Ramioul, H., and Deltour, G., *Bull. Acad. Royale Med.*, 1952, v17, 460.

Received April 21, 1953. P.S.E.B.M., 1953, v83.

A Filterable Agent, Recovered from Ak Leukemic Extracts, Causing Salivary Gland Carcinomas in C3H Mice.* (20376)

LUDWIK GROSS.

From the Cancer Research Unit, Veterans Administration Hospital, Bronx, New York.

In previous experiments reported from this laboratory, Seitz filters were used for the filtration of Ak leukemic extracts; the results, however, did not appear to be entirely satisfactory, since only a comparatively small number of C3H mice developed leukemia following inoculation with such filtered extracts (1). The possibility had to be considered that much of the leukemic agent might have been absorbed, and thus retained, by the asbestos pads used in Seitz filters. For that reason a series of experiments has been carried out in which, instead of Seitz, the Selas† microporous porcelain filter candles have been used for filtration of leukemic extracts. Since the leukemic extracts became more active following centrifugation at 7000 x g(2), such centrifugation was carried out, in this study, prior to filtration of the extracts. Newborn (less than 16 hours old) C3H or C3H(f) mice were inoculated with such filtered extracts, and were then observed for development of leukemia. The results were surprising. Some of the inoculated mice developed typical generalized leukemia. Others, however, developed bilateral carcinomas in the cervical region, arising probably in the salivary glands.

Materials and methods. Leukemic extracts. Mice of the Ak line that developed leukemia either spontaneously, or as a result of transplantation of Ak leukemic cells, were sacrificed by ether inhalation; the livers, spleens, mesenteric tumors and peripheral lymph glands were removed aseptically, and ground for 2 to 3 minutes in a porcelain mortar, chilled (+4°C) physiological solution of sodium chloride added in sufficient quantity to obtain cell suspensions of 20% concentration. These leukemic cell suspensions were

---

* Aided, in part, by a grant from the Damon Runyon Memorial Fund for Cancer Research.

† Selas Corp. of America, Philadelphia, Pa.

TABLE I. Results of Inoculations of Filtered (Selas) Ak Leukemic Extracts into Newborn C3H or C3H(f) Mice.

							Fresh[‡] fil. extr.				Heated (68°C ½ hr) fil. extr.	
No. of individual exp.	Prelim. centrif.,[*] 7000 × g, min.	Filter candle (Selas) porosity	Filter pressure[†]	Strain of mice inoc.	Avg age at inoc., hr	Sex	No. of mice inoc.	No. of mice dev. neck tum.	No. of mice dev. leuk.	Avg age when neck tum. (leuk.) appeared, mo	No. of mice inoc.	No. of mice dev. neck tum. or leuk.
5	5 to 10	03	pos	C3H	<12	♀	7	2	2	3.6	6	0
						♂	5	1	0		5	0
5	"	"	"	C3H(f)	< 9	♀	7	2	0	3.8	3	0
						♂	7	1	2		5	1[§]
9	10	"	vac	C3H	<11	♀	10	4	1	4.5	5	0
						♂	9	5	0		9	0
11	5 to 10	"	"	C3H(f)	<12	♀	17	0	2	3.8	7	0
						♂	13	0	0		11	0
1	10	02	"	C3H	<16	♀	2	0	1	3	2	0
						♂	1	0	0			
2	"	"	"	C3H(f)	"	♀	3	0	1	4	1	0
						♂	3	0	0		3	0
				Total			84	15	9		57	1[§]

[*] Ak mice which developed ''spontaneous'' leukemia were used as donors for preparation of leukemic extracts in 16 experiments. In all other (17) experiments Ak mice with transplanted leukemia were used. Leukemic extracts centrifugated in all experiments at 3000 RPM (1400 × g) for 15 min. prior to centrifugation at 7000 × g indicated in this column.

[†] Positive (10 lb) pressure (95% N mixed with 5% CO_2) used for filtration of leukemic extracts through Selas candles in experiments designated in this column ''pos.'' Experiments designated ''vac,'' vacuum pressure of 20-25 mm of mercury was applied.

[‡] Filtered leukemic extracts used for inoculation immediately after preparation in 15 experiments. Extracts kept in ice water at 0°C prior to use for inoculation for less than 7 hr in 4 exp., for less than 24 hr in 10 exp., and for less than 48 hr in 4 exp.

[§] This mouse developed a neck tumor at 4 mo of age.

centrifuged at 0°C in an International PR-1 Centrifuge at 3000 RPM (1400 x g) for 15 minutes. The supernatant was then removed, placed in a chilled (0°C) celluloid tube, and centrifugated at 0°C, at 9500 RPM (7000 x g) for periods of time varying from 5 to 10 minutes (Table I) in the International PR-1 Centrifuge. It required 2 to 3 minutes for the head of the high-speed attachment to reach 7000 x g. After centrifugation at this speed for the specified time (Table I), the motor was turned off, the pulley disconnected, and the centrifuge head came gradually to a standstill (requiring additional 7 to 10 minutes). Approximately two-thirds of the upper part of the resulting supernatant was then carefully removed with a syringe, and immediately passed through the filter, as described below.

Filtration. Selas[†] microporous filter candles with filter elements 2" long and of a 5/8" diameter, porosity 03 (FPS-52-03), and in a few instances of porosity 02 (FPS-52-02), were used (Table I). In these candles, the filter elements are glazed directly to the porcelain connectors, forming single units, without any metal or rubber connections. The filter candles were inserted through a rubber stopper into standard, 125 cc (or 250 cc) pyrex vacuum flasks, with the filter element placed downward, inside the flask, and the porcelain connector extending, through the stopper, outside the top of the flask. The centrifugated leukemic extracts, ready for filtration, were then introduced, by means of a sterile syringe, directly inside of the porcelain connector extending outside the flask (Fig. 1). Vacuum pressure of approximately 20 to 25 mm mercury was then applied, and the extract passed through the filter into the flask. The extract did not contact with the rubber tubing or any other part of the filtration system except the filter candle and the pyrex flask. In a few experiments (Table I), positive pressure, instead of vacuum pressure, was applied (mix-

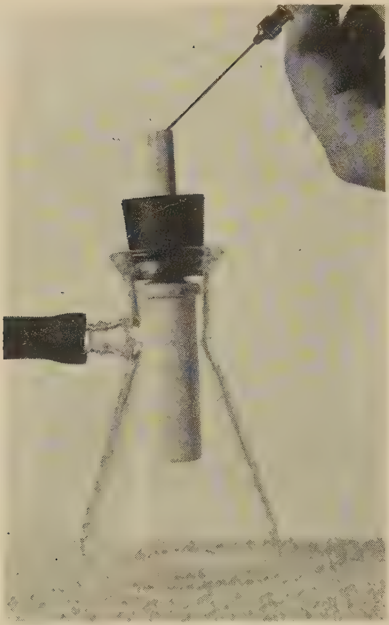


FIG. 1. Selas filter candle mounted for vacuum filtration. The leukemic extract is introduced directly, by means of a syringe, into the porcelain connector of the filter candle extending outside the flask.

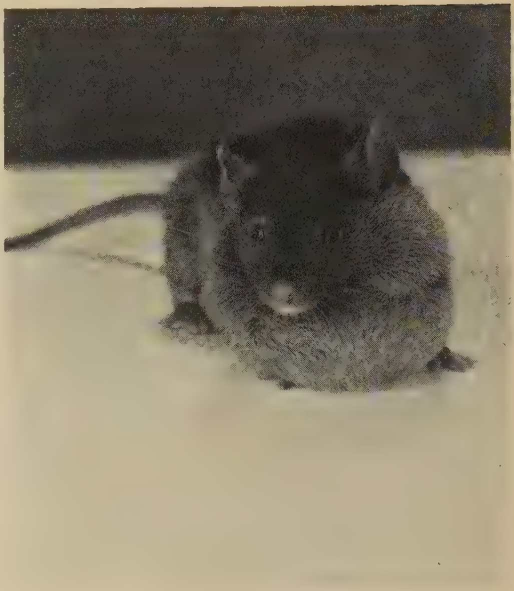


FIG. 2. Salivary gland epithelioma developed in a C3H male at 4 mo of age. This mouse had been inoculated with Ak leukemic filtered extract (Selas candle porosity 03) within 12 hr after birth. Photograph taken after tumor had been allowed to grow for 1 mo.

ture of 95% nitrogen, and 5% carbon dioxide, under 10 lb pressure, blown into the filter candle through a rubber tubing pulled over the porcelain connector). Each filter had a record number, and was first tested with, and found to be impervious to, *E. coli.* The fresh suspension of *E. coli* culture in tryptose phosphate broth, diluted 1:10 with physiological saline solution, was passed through the filter candle, and the resulting filtered extract proved to be sterile, as evidenced by inoculation of tryptose phosphate broth media. After this preliminary test, the filter candle was washed with distilled water, autoclaved (one hour at 115°C at 15 lb) and used for the filtration of the leukemic extracts. Immediately after filtration, the candle was again tested with, and found to be impervious to, *E. coli.* The candle was now baked in a Selas filter candle furnace, in order to burn out organic matter remaining after filtration, then washed with distilled water, and autoclaved. For the second time the candle was now used for filtration of another leukemic extract; immediately afterwards, the candle was again

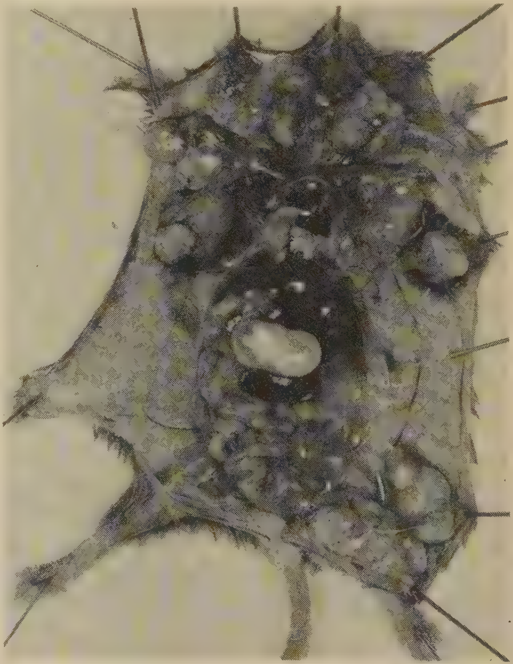


FIG. 3. This C3H(f) female was inoculated (within 16 hr after birth) with a centrifugated (7000 × g) extract prepared from normal Ak embryos, and developed bilateral salivary gland carcinomas at 3 mo of age. Two weeks later, additional tumors developed in both axillary pits, and in the left groin.

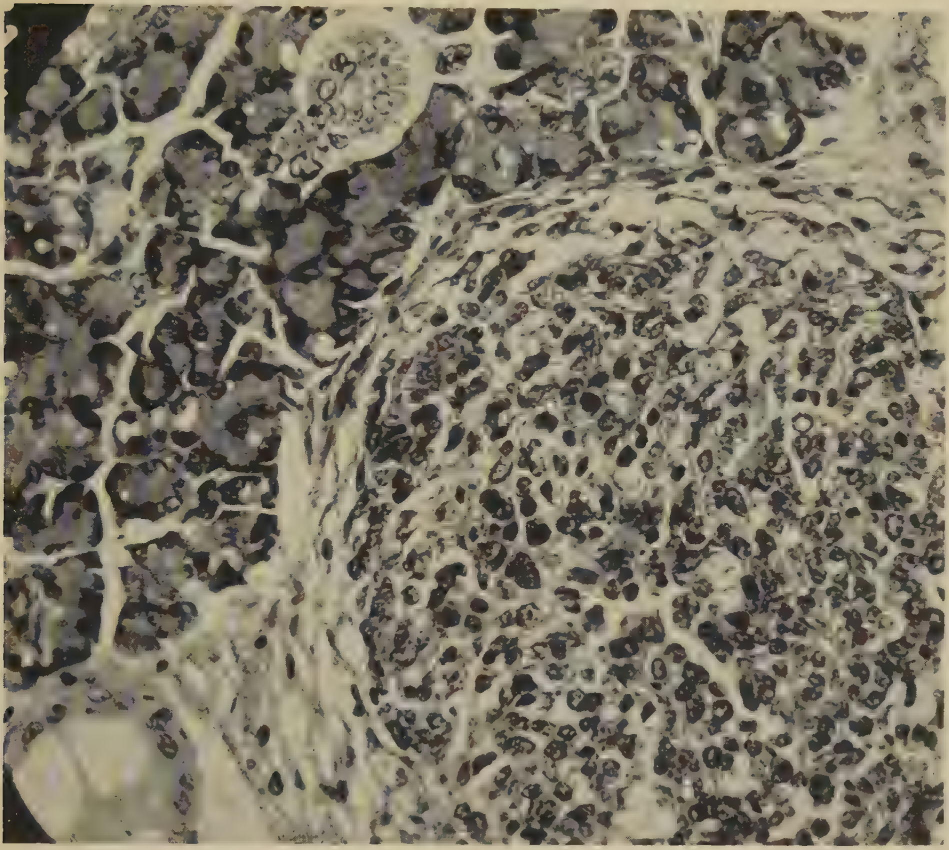


FIG. 4. Photomicrograph of a salivary gland epithelioma, most probably arising in the parotid gland. This tumor developed in a C3H female, at 3½ mo of age, following inoculation (within 12 hr after birth) of a filtered (Selas, porosity 03) Ak leukemic extract. (H. & E. × 475.)

tested with, and found to be impervious to, *E. coli*. The procedure of baking in an oven, washing, and autoclaving, was then repeated, and again the candle was used for the filtration of a third leukemic extract, followed immediately by testing with *E. coli*. After 4 or 5 of these consecutive filtrations, the candle was discarded.

The filtration procedure was carried out as follows: The autoclaved Selas filter candle was first briefly washed by passing 10 to 15 cc of chilled (0°C) sterile physiological saline solution through the candle, under the usual vacuum pressure of 20 to 25 mm of mercury. The leukemic supernate resulting from the 7000 x g centrifugation was then passed through the filter candle using either positive or vacuum pressure. Immediately after filtration, the filtered leukemic extract was removed from the flask aseptically, placed in a sterile pyrex glass tube immersed in ice-water, and designated "fresh filtered leukemic extract." Part of the filtered extract was heated in an electric waterbath to a temperature of 68°C for 30 minutes (Table I), then placed in a sterile pyrex glass tube immersed in ice-water, and designated "heated filtered leukemic extract." The extracts were inoculated in most instances immediately or within 24 hours, occasionally, however, within 48 hours, after their preparation (Table I), having been kept in tubes immersed in a container filled with ice tubes, and placed in an electric refrigerator.

Inoculation of mice. Newborn (less than 16 hours old) mice of our colonies(1,2) of either the C3H line or of the foster nursed C3H(f) subline, bred in our laboratory by brother-to-sister mating, and having an incidence of spontaneous leukemia far below 0.5%, were then inoculated with the extracts.

Each newborn litter was divided, one part inoculated with the fresh, and the other part with heated filtered extract. Inoculations were subcutaneous (0.1 cc each). The skin punctures were dried off gently with a sterile sponge, and then sealed off with a drop of collodion. For the purpose of identification, the mice of each litter injected with either the fresh or the heated supernate had their tails tied off at half length and cut immediately after inoculation. The mice were weaned at 21 days of age; they were then identified by individual numbers, and separated by sex.

Results. The results were surprising: while only 9 of the 84 mice that had been inoculated with the fresh filtered extracts developed generalized leukemia, 15 other mice developed, at an average age of 3.3 months, bilateral tumors in the cervical region (Fig. 2). These tumors slowly enlarged, and eventually formed enormous confluent masses around their necks. On dissection (Fig. 3) these tumors presented grape-like clusters of individual small tumors, or, in more advanced cases, solid masses of hard (difficult to cut and grind) white tumor tissue, infiltrating the cervical region, on both sides of the neck. Microscopic examination of the tumors (Fig. 4) revealed undifferentiated tumor cells with large, vesicular nuclei, and numerous atypical mitotic figures, frequently growing either in bands of cells, or forming glandular pattern; occasionally, however, no pattern was discernible, the tumor cells growing chaotically (Fig. 5). In spite of their individual differences, these tumors, apparently arising in the salivary glands, could be classified as carcinomas.‡ Additional tumors could sometimes be found in the axillary pits, and occasionally also in the inguinal region (Fig. 3). Animals with these tumors showed no other signs of disease: microscopic sections of liver showed no changes; the peripheral blood picture as well as the bone marrow were normal. The cervical tumors reported in this study were essentially similar to those recently observed in C3H mice following inoculation of centrifugated (144000 x g) Ak leukemic extracts(3). The neck tumors could be transplanted, by cell inoculation, into mice of the C3H, but not those of the Ak, line(3,4). They were found to grow in some of the inoculated C3H mice, after an incubation period of 4 to 8 weeks, at the site of implantation (subcutaneous or intraperitoneal), infiltrating the surrounding tissues and eventually forming enormous tumors.

That the neck tumor agent could be inactivated by heating to 68°C for 30 minutes, was evident from a series of experiments (Table I), in which 54 control litter-mates were inoculated with the heated leukemic ex-

---

‡ Dr. Maurice N. Richter, Department of Pathology, New York University-Bellevue Medical Center, kindly reviewed the tumor slides, and commented as follows: The sections of neck tumors suggest that the tumor is in the salivary glands, often forming cell accumulations around small glands or ducts, and eventually displacing most of the glandular tissue. In the less involved areas, small groups of tumor cells suggest the possibility of multiple foci of origin. The cells are rounded or elongated. I am inclined to believe that all of the cells are epithelial. It is possible that these tumors arise from myoepithelial cells of the salivary glands, as suggested by Lippincott and associates(6). However, I have been unable to see longitudinal fibrils, and consider the origin uncertain at the present time.

Dr. Jacob Furth, Biology Division, Oak Ridge National Laboratory, dissected in our laboratory two C3H mice that had developed bilateral neck tumors as a result of inoculation with Ak leukemic filtered (Selas) extracts. Dr. Furth also reviewed the microscopic slides of these tumors. His comments follow: "The 'neck' tumors in the two animals which I have dissected involve the parotid gland and extend in the region of the submaxillary glands, but the latter were free from tumor. Likewise the lymph nodes of these animals examined showed neither leukemic involvement nor metastatic tumor. The site of the primary tumors is the parotid gland. The sections which you have shown me indicate multicentric origin of the tumors in the parotid glands. The appearance of the tumors in sections, some resembling carcinomas, others sarcomas support the belief that they originate in a salivary gland. Further dissections are indicated to find out if salivary glands other than the parotid can give rise to primary tumors. Whether the subcutaneous tumors in the axilla and groins are primary or secondary remains to be determined; if primary, the possibility exists that your agent can also cause cancer in the mammary gland. I recommend designating the 'neck' tumor as 'salivary gland tumor'."

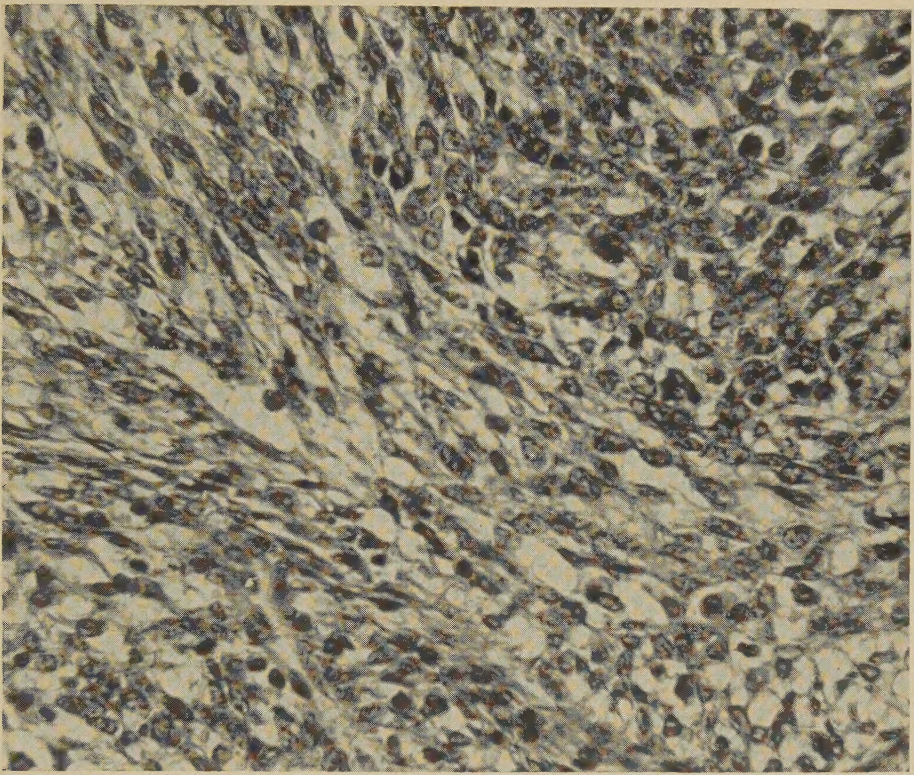


FIG. 5. Photomicrograph of a salivary gland tumor showing a sarcomatous appearance. Elsewhere same tumor had structure of anaplastic adenocarcinoma, particularly on opposite side of neck in same mouse. This tumor developed bilaterally on the neck of a C3H female, at 3 mo of age, following inoculation (within 16 hr after birth) of a filtered (Selas, porosity 03) Ak leukemic extract.

tracts: only one of them developed a salivary gland tumor.

The presence of the tumor agent in normal Ak embryos. In order to determine whether the salivary gland tumor agent is present in normal Ak embryos, 2 young (3-month-old) healthy pregnant Ak females were sacrificed, their embryos removed aseptically, separated from placenta, and ground with physiological saline solution. The resulting embryo cell suspension was centrifugated at 3000 RPM (1400 x g) for 15 minutes; the supernate was again centrifugated at 9500 RPM (7000 x g) for 5 minutes. The final supernate was removed carefully with a syringe, and immediately inoculated into 4 newborn (less than 16 hours old) litters of C3H(f) mice. The litters were divided as follows: 12 infant mice were inoculated with the Ak embryo centrifugated extract, and 11 control litter-mates were inoculated with a similar extract prepared simultaneously under identical experimental conditions from C3H embryos. As a result, 4 of the 12 C3H(f) mice that had been inoculated with the Ak embryo extracts developed typical, bilateral neck carcinomas at 3 months of age; the 11 control litter-mates that had been inoculated with C3H embryo extracts remained in good health. In previous experiments(5), the inoculation of Ak embryos resulted in the development of a generalized leukemia in the injected C3H(f) mice, and not that of neck tumors; however, in previous studies, Ak embryo cell suspensions, and not centrifugated extracts, had been used for inoculation(5).

Discussion. It is apparent from experiments reported in this and previous(1-3) studies, that the inoculation of centrifugated, or filtered Ak leukemic extracts into newborn (less than 16 hours old) C3H or C3H(f) mice may result in the development of either a generalized leukemia, or bilateral cervical epitheliomas, possibly arising in the salivary glands.‡ One may determine, by selecting proper experimental conditions, which of the two neoplastic conditions, *i.e.* leukemia or cervical epitheliomas will develop in the inoculated animals. When Ak leukemic extracts centrifugated at 3000 RPM (1400 x g), or 9500 RPM (7000 x g), were inoculated into newborn C3H or C3H(f) mice, leukemia resulted and not salivary gland tumors(1,2). When Ak leukemic extracts were filtered through Berkefeld (N) candles, then inoculated into newborn C3H mice, leukemias developed with only very few mice developing cervical epitheliomas(3). On the other hand, when the Ak leukemic extracts were centrifugated at 144000 x g and the resulting supernatant was used for inoculation of newborn C3H mice, cervical epitheliomas developed much more frequently than generalized leukemia(3). In experiments reported in this study the Ak leukemic extracts were filtered through Selas micro-porcelain filter candles, and the extracts were inoculated; as a result, 15 out of 84 inoculated animals developed cervical epitheliomas, and only 9 generalized leukemia. Curiously, only extracts that had been filtered through candles having a very fine porosity of 03, produced neck carcinomas (but occasionally also leukemia); of 9 mice inoculated with extracts filtered through 02 porosity candles, 2 developed leukemia, but none salivary gland tumors (Table I). The number of mice in this series is too small to draw any definite conclusions, and additional experiments have since been instituted(4) to determine whether the finer porosity filter candles (03) must be used for the preparation of extracts capable of producing salivary gland epitheliomas.

Leukemic extracts could be inactivated by heating in a waterbath to 68°C for 30 minutes. Of 54 control litter mates inoculated with such heated extracts, one developed a salivary gland tumor (Table I). There is a possibility, that in this experiment the tube containing the extract had not been completely immersed in the waterbath, and that part of the extract adhering to the top of the test tube, might not have been properly heated and remained active (this technical error has been corrected in subsequent experiments). On the other hand, it is also possible that the neck tumor agent may require heating to a temperature slightly higher than 68°C for 30 minutes for complete neutralization.

While the number of leukemias developing among the mice that had been inoculated with fresh filtered extracts is small (9 out of 84), it must be stated that only 7 months have elapsed since these experiments were initiated; some of the injected mice are only 5 months old at the time of this writing, and may develop leukemia later; moreover, 15 mice developed salivary gland tumors at 3.3 months of age, that is, before they had a chance to develop leukemia.

On rare occasions, salivary gland tumors have been recorded in albino mice of strains C and A(6). However we have not seen salivary gland tumors develop "spontaneously" in untreated mice of either the C3H line or of the C3H(f) subline. We have never seen salivary gland epitheliomas in mice of our colony of Ak mice, which were donors of the leukemic extracts. Moreover, among the C3H or C3H(f) mice that had been inoculated with filtered, or with centrifugated Ak leukemic extracts(1-3), some developed either leukemia, or cervical carcinomas(3), but never both.

The following working hypothesis may explain the experimental results thus far obtained: Mice of the Ak line may carry at least 2 different oncogenic agents, both present in leukemic Ak extracts. One of these agents would cause leukemia ("Ak leukemic agent"); the other salivary gland epitheliomas ("Ak tumor agent"). Since these epitheliomas do not spontaneously develop in mice of the Ak line, whereas spontaneous leukemia is very common in these animals, it would follow that in mice of the Ak line the leukemic agent interferes with the tumor agent, or, that the Ak mice are not susceptible to the pathogenic action of the Ak tumor agent, even though they carry, and transmit it from one generation to another, along with the Ak leukemic agent(4,5). Experiments reported in this, and the preceding(3) article could be explained by the assumption that 2 such different agents are harbored by mice of the Ak line, and can be extracted from organs of leukemic Ak mice. These agents could be separated either by (a) ultracentrifugation (144000 x g), the carcinoma agent remaining mainly in the supernate(3), or (b) by filtering the extracts through fine porosity filters, such as Selas 03. Heating to 63°C or 64°C for 30 minutes may also neutralize the leukemic agent, but not the agent causing salivary gland epitheliomas(4). A higher temperature (at least 68°C for 30 minutes) may be required for complete inactivation.

Should leukemia be caused by a transmissible agent not only in chickens and mice, but also in humans, the possibility would have to be considered that in humans also the leukemic agent may be accompanied by another oncogenic agent transmitted, along with the leukemic agent, from one generation to another. An interference phenomenon could be responsible for the apparent latency of either, or both, in most of the carrier hosts. Occasionally, however, a segregation of such agents may occur, resulting in the development of leukemia in some, and carcinomas in others. This hypothesis may offer an explanation of clinical observations suggesting that in families of patients suffering from leukemia, cancer is more common than in the average population(7,8).

Summary. 1. Extracts prepared from livers, spleens, and lymphatic tumors of leukemic Ak mice, were centrifugated at 1400 x g (15 minutes), then 7000 x g (5 to 10 minutes), and finally passed through Selas porcelain filters. The filtered extracts were then inoculated into newborn (less than 16 hours old) C3H or C3H(f) suckling mice. 2. Of the 84 mice inoculated with fresh, filtered extracts, 9 died from leukemia, and 15 others developed bilateral epitheliomas possibly arising in the salivary glands.‡ Of the 54 control littermates inoculated with heated (68°C for 30 minutes) extracts, only one developed a cervical tumor. 3. Extracts prepared from Ak leukemic cells may therefore contain 2 different agents, pathogenic for newborn C3H or C3H(f) mice: one, causing leukemia, and another salivary gland epitheliomas. These agents may differ in size. 4. It is assumed that the leukemic agent may interfere with the tumor agent preventing it from exerting its pathogenic properties on the cells of its usual Ak, carrier host, or that mice of the Ak line may be naturally resistant to the tumor agent. 5. Salivary gland tumors have not been observed in untreated mice of either the Ak, the C3H, or C3H(f) inbred lines.

---

1. Gross, L., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 342.

2. ———, *Cancer,* 1953, v6, 153.

3. ———, Cancer, 1953, v6, (Sept.) in press.

4. ———, Experiments to be published.

5. ———, PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 27.

6. Lippincott, S. W., Edwards, J. E., Grady, H. G., and Stewart, H. L., *J. Nat. Cancer Inst.,* 1942, v3, 199.

7. Gross, L., and Matte, M. L., *N. Y. State J. Med.,* 1948, v48, 1283.

8. Videbaek, A., *Heredity in Human Leukemia,* H. K. Lewis & Co. Ltd., 1947, pp. 279.

Received June 8, 1953. P.S.E.B.M., 1953. v83.